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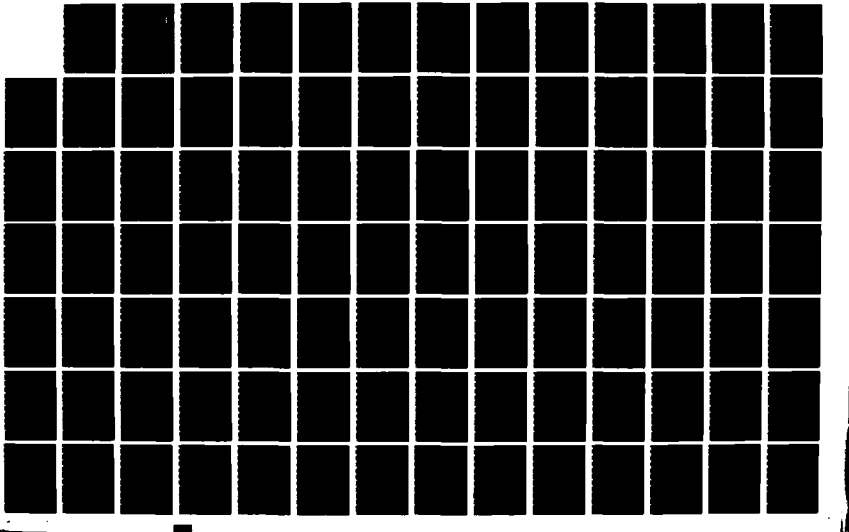

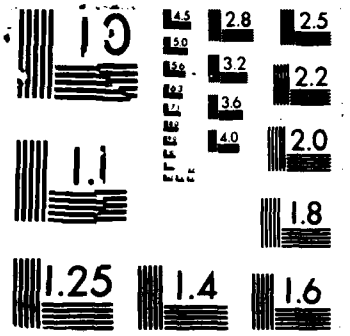
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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Techniques were developed and/or improved for the study of dengue viruses in their mosquito vectors. Improved techniques for infection of mosquitoes with dengue viruses, for in vitro assay of transmission, and for titration of infected mosquitoes were developed. The titration technique, using microtiter plates and indirect fluorescence, proved to be a fast, reliable, and inexpensive technique for titration of dengue viruses in mosquitoes. Studies were also conducted to determine the extent and phlogenetic range of interference to superinfection in Aedes albopictus mosquitoes previously infected with selected dengue viruses.

Studies were conducted to compare the efficiency of oral infection, of replication, and of oral transmission of dengue parent and derived candidate vaccine viruses in potential vector mosquitoes. Candidate vaccine viruses were also monitored for phenotypic stability to ensure that they did not revert to virulence as a consequence of passage

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19. Abstract

in the mosquitoes. None tested reverted to parental phenotype in these studies.

Results of the vaccine studies are summarized below:

1. Dengue-1 The dengue-1 candidate vaccine virus (TP-56) was not altered in its ability to infect and to replicate in Aedes aegypti or Aedes albopictus mosquitoes.
   The dengue-1 candidate vaccine virus (45AZ5) was similar to the parent virus in its ability to infect and to replicate in the two mosquito species.

2. Dengue-2 The dengue-2 candidate vaccine virus (PR-159/S-1) was significantly less efficient than the parent virus in oral infection, in dissemination, and in transmission by both mosquito species. It did not revert to parental phenotype during mosquito passage.

3. Dengue-3 The dengue-3 candidate vaccine virus (CH53489, clone 24/28) did not diffe from the parental virus it its ability to infect the two mosquito species. Interesting, neither the parent nor the vaccine virus were efficiently transmitted by the mosquitoes.

4. Dengue-4 The dengue-4 candidate vaccine virus (H-241, lot L) was less efficient than its parent virus in infection and in replication in Aedes albopictus mosquitoes.

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Annual and Final Report

Barry J. Beaty, Ph.D.

July 1, 1986

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Summary

Techniques were developed and improved for the study of dengue viruses in the mosquito vector. A technique for oral infection using freshly prepared virus stocks proved to be efficient. An improved in vitro assay for transmission of dengue parent and vaccine viruses was developed and was assessed. Using an oil-charged capillary feeding system, saliva can rapidly and reliably be collected from even moribund mosquitoes.

As fast, reliable, and inexpensive method was developed for titration of dengue viruses in microtiter plates using an indirect fluorescent-antibody technique. Virus titers obtained by the microtiter plate system were not statistically different ($P<0.05$) than those obtained by the multichambered slide system.

To determine if viral interference occurs during dual infection with dengue viruses, Aedes albopictus mosquitoes were intrathoracically inoculated with small plaque, temperature sensitive, candidate vaccine viruses and then challenged with homologous dengue parent strains or with heterologous viruses. From the limited studies conducted, interference to superinfection between the dengue viruses in vectors is dependent upon the combination of viruses used.

Studies were conducted to compare the efficiency of oral infection, of replication and of oral transmission of dengue candidate vaccine viruses and their respective parent viruses in vector mosquitoes.

The dengue-1 candidate vaccine virus (TP-56) was not altered in its ability to infect and to replicate in Aedes aegypti or Ae. albopictus mosquitoes. Oral infection rates with the vaccine virus were as high or higher for the parent virus. Further, the vaccine virus replicated more efficiently in mosquitoes after oral infection than the parent virus.

The dengue-1 candidate vaccine virus (45AZ5) was similar to the parent virus in its ability to infect, to replicate in Aedes aegypti or Aedes albopictus mosquitoes. Oral infection rates with the vaccine virus were as high or higher when compared to the parent virus.

The dengue-2 candidate vaccine virus (PR-159/S-1) was less efficient than the parent virus in interactions with potential vector species. The vaccine was less efficient in oral infection of mosquitoes, in dissemination of virus, and in transmission of virus. The attenuated virus remained temperature sensitive after replication in mosquitoes. Plaque sizes were heterogeneous, however, no large plaques were seen.

The dengue-3 candidate vaccine virus (CH53489, clone 24/28) was not modified in its ability to orally infect either Aedes albopictus or Aedes aegypti mosquitoes. However, neither parent or vaccine viruses were efficiently transmitted despite high dissemination rates. The attenuated virus remained temperature sensitive and of small plaque morphology after replication in mosquitoes.

The dengue-4 candidate vaccine virus (H-241, Lot 1) was less efficient than its parent virus in infection and replication in Aedes albopictus mosquitoes.

In general, increases in extrinsic incubation temperature of dengue virus infected mosquitoes tended to increase replication of the virus resulting in the reduction or elimination of the eclipse period and an increase in peak virus titers.

Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Body of the Report

I. Statement of the Problem

The purpose of this research project was to determine if dengue parental and candidate vaccine viruses differ in their respective abilities to infect, to replicate in, and to be transmitted by Aedes aegypti and Aedes albopictus mosquitoes. Attenuated candidate vaccines and parental strains of each of the four types of dengue virus were compared in their vector-virus interactions.

The second, and related, objective of this research project was to determine if attenuated vaccine strains reverted to virulence after mosquito passage. Should a live dengue vaccine be capable of infecting and subsequently be transmitted by mosquitoes to a susceptible vertebrate and should the vaccine revert to virulence as a consequence of mosquito passage, then a natural infection cycle could be initiated.

The rationale for this project was that the temperature sensitive (ts) vaccine strains of the dengue viruses that were attenuated for man were also modified in one or more parameters of vector-virus interactions. The hypotheses were 1) the vaccine strains would be less capable than parent strains in vector infection, 2) vaccine strains would differ from parent strains in their mode of development, 3) the vaccine strains would be less efficiently transmitted than parent strains, and 4) that the small plaque, ts mutant virus populations would remain stable upon passage in vector mosquitoes.

II. Background

Dengue is of great tactical significance to the military because large numbers of troops can become incapacitated in a short period of time. Attenuated dengue vaccines were developed through programs of Walter Reed Army Institue of Research (WRAIR) as a mechanism to preclude the problems the viruses cause.

Ideally an arbovirus vaccine should not produce viremia, but if it does, it is reasonable to expect that the vaccine strain will infect mosquitoes poorly and will be inefficiently transmitted. This was demonstrated with the 17D yellow fever vaccine (Roubaud et al., 1937; Whitman, 1939), French neuroptropic yellow fever vaccine (Davis et al., 1932; Roubaud and Stefanopoulo, 1933; Peltier et al., 1939), mouse-adapted dengue type 1 (Sabin, 1948), African green monkey kidney-adapted dengue type 2 (Price, 1973), and attenuated Japanese encephalitis vaccine virus (Chen and Beaty, 1982). Sabin (1948) showed that attenuated dengue, passed through mosquitoes, did not revert to pathogenicity for man. Chen and Beaty (1982) demonstrated that the attenuated Japanese encephalitis vaccine did not revert to mouse virulence after mosquito passage, nor did mosquito passage result in phenotypic changes for the candidate dengue-2 vaccine (Miller et al., 1982).

Thus, even if these vaccine viruses did develop sufficient viremia to infect vectors, there would be little likelihood that the virus would be transmitted and would revert to virulence.

III. Approach

The working hypothesis was made that the ts candidate vaccine viruses and the parental wild-type viruses would behave differently in vector mosquitoes. To test this hypothesis the efficiency of oral infection of each parent and vaccine candidate strain was to be determined in dose response studies. Sequential 10-fold dilutions of the virus preparations were used to infect groups of a minimum of 10 sibling mosquitoes per dilution. Such studies would also provide information about the optimal infective dose for the transmission and pathogenesis studies; doses much greater than the threshold could obscure differences in infectivity between the vaccine and parent viruses.

To determine infection rates, extrinsic incubation periods, and rates of oral transmission, mosquitoes were to be infected via engorgement on known titer blood-virus mixtures. Vector competence studies and especially dose-response studies were greatly facilitated by the development of an efficient technique to orally infect Aedes mosquitoes using artificial blood meal systems (Miller et al., 1982).

Vector-virus interactions were to be further investigated using immunofluorescent (IF) techniques to localize antigen in situ in organ dissections and cryostat sections of infected mosquitoes. The sites of restriction of replication (if restriction exists) of the vaccine strains would be defined by the comparative IF studies of antigen development in organs of mosquitoes.

A further obstacle to assessment of vector competence was the lack of a suitable laboratory animal to use to detect mosquito transmission of low passage or attenuated dengue viruses. Development of an in vitro assay which permitted assay of transmission by inoculation of collected mosquito saliva into recipient mosquitoes was a major advance (Beaty and Aitken, 1979). This technique facilitated transmission assays for viruses that did not cause observable morbidity or mortality in animals. Unfortunately, mosquitoes could not always be induced to engorge upon the artificial meal system used to capture the saliva. Refinement by Rossignol and Spielman (1982) of a saliva capture technique using oil-charged capillaries (Hurlbut, 1966), provided a new in vitro technique to assay for virus transmission.

This combination of transmission, comparative pathogenesis studies, and the determination of dose-response curves (thresholds of infection) were thought to be adequate to reveal differences in vector-virus interactions between parent and vaccine viruses.

IV. Materials and Methods

A. Viruses

The attenuated candidate vaccine viruses were developed from parent strains isolated from infected human serum. The parent strains were attenuated by multiple passages in cell culture and clonal dilution until a stable virus clone that produced small plaques in LLC-MK_2 cells and that was temperature sensitive was produced. The parent and vaccine viruses were received from WRAIR as lypholized or frozen aliquots. For the studies reported here the virus stocks were grown in an established line of Ae. albopictus C6/36 cells for 14 days at 27°C, aliquoted, and stored at -70°C.

B. Mosquitoes

Two vector species were used in these studies: the Puerto Rico strain of Ae. aegypti and the Oahu (Hawaii) strain of Ae. albopictus. Emerged adults were allowed to feed on sugar cubes and had access to water wicks. When this procedure was followed it was not necessary to starve mosquitoes prior to engorgement on infectious blood meals; the majority rapidly engorged to repletion. Only mosquitoes that became fully engorged were used in experiments. The engorged mosquitoes were maintained at either 27°C, 30°C, or 33°C and at approximately 75% relative humidity (RH).

C. Conjugate

Antibodies for the anti-dengue conjugates were prepared by hyperimmunization of mice (Brandt et al., 1967). Immunoglobulins were precipitated from the ascitic fluids with $(NH_4)_2SO_4$ and conjugated with fluorescein isothiocyanate (Spendlove, 1966; Hebert et al., 1972). Conjugated antibodies were purified by Sephadex G-50 column chromatography.

D. Virus Assay

Titrations. All titrations, with the exception of those involving dengue-3 viruses, were done using Lab-Tek slides seeded with Ae. albopictus C6/36 cells. Serial 10-fold dilutions of the preparations were inoculated into slides. After 7 days incubation (28°C), they were examined by IF for the presence of viral antigen. Because of the considerable expense associated with titeration of mosquitoes in Lab-Tek slides, an alternate technique using 96 well microtiter plates was developed (Schoepp and Beaty, 1984). The development of this technique will be detailed later.

Antigen detection. Immunofluorescence was used to localized viral antigen in situ in organ dissections and cryostat sections of mosquitoes (Beaty and Thompson, 1976, 1978) and in head and abdominal squash preparations (Kuberski and Rosen, 1977).

E. Efficient Oral Infection of Mosquitoes

Considerable effort was devoted to development of an effective technique for oral infection of mosquitoes with low passage or vaccine strains of dengue virus. Defibrinated blood-virus preparations are known to be much less efficient than a viremic host in mediating midgut infection. The artificial meal must be several logs higher in titer in order to obtain the same infection rate. Studies were conducted to assess the ability of alternate blood sources and preparations as well as different virus preparations to infect mosquitoes. These included: 1) the use of blood from vertebrates other than rabbits, 2) the use of chemically defibrinated instead of mechanically defibrinated blood, 3) the use of viremic suckling mice or guinea pigs, 4) the use of unfrozen, dengue-infected leukocytes, and 5) the use of unfrozen, dengue-infected LLC-MK_2 or Ae. albopictus C6/36 cells.

After considerable experimentation, the following protocol was developed: Parent and vaccine viruses each were inoculated into flasks of Ae. albopictus C6/36 cells (28°C). After incubation periods of 14-17 days, cells were detached from the flasks with rubber policemen, and the cell fluid suspensions

were centrifuged at 500xg for 10 minutes. The cell pellet was resuspended in 2 ml of the remaining supernatant fluid, and combined with 2 ml of washed human or rabbit red blood cells and 2 ml of 10% sucrose in heat-inactivated calf serum. Drops of this artificial blood meal were placed on the screening of cages holding mosquitoes. Engorged mosquitoes were removed and maintained at various temperatures and 75% RH for 14-21 days.

F. Threshold of Infection (Dose-Response Studies)

To determine the comparative thresholds of infection for the parent and vaccine strains in Ae. albopictus and Ae. aegypti, mosquitoes were permitted to engorge on 10-fold dilutions of infectious blood meal preparations as described above. After 21 days extrinsic incubation at 27°C, mosquito heads and abdomens were severed, squashed on microscope slides and examined by IF for the presence of viral antigen. Detection of viral antigen in abdominal tissues indicated that the midgut had become infected. Detection of antigen in head tissues indicated that the virus had escaped the midgut and had disseminated to secondary target organs. The titer of each parent and vaccine virus preparation necessary to infect 50% of the engorging mosquitoes (median oral infectious dose - OID_{50}) was calculated by the method of Karber (1931).

G. In vitro Oral Transmission Assay

This laborious technique was necessitated by the lack of a small laboratory animal model susceptible to the low passage dengue viruses and attenuated candidate vaccine viruses.

After 14-24 days extrinsic incubation, mosquitoes were starved overnight prior to the transmission attempt. Capillaries were charged with a 10% FBS-sucrose solution. Mosquitoes were cold anesthetized, wings and legs were removed, and the proboscis was inserted into the capillary tube. Mosquitoes were allowed to engorge for 1 hour before they were removed, and heads and abdomens were severed and squashed on slides. The smears were stained with the anti-dengue conjugate and examined for the presence of viral antigen by IF. After engorgment the contents of the meal were promptly inoculated intrathoracically into 10 recipient mosquitoes. After 10-14 days incubation, recipient mosquitoes were processed by the head squash IF procedure (Kuberski and Rosen, 1977).

H. Improved In vitro Oral Transmission Assay

Attempts were made to improve the in vitro transmission technique using oil-charged capillaries. Legs and wings were removed from mosquitoes and probisci were inserted into capillary pipettes charged with 3.5 ul of Cargille immersion oil. The proboscis of each mosquito was inserted into the oil, forcing the mosquito to salivate. After 60 to 90 minutes exposure, mosquitoes were removed and the capillaries were placed in microcentrifuge tubes containing 0.1 ml of 20% FBS-PBS diluent. Tubes were centrifuged to force virus into the diluent, which was subsequently inoculated into recipient mosquitoes. After incubation at 27°C for 14 days the recipient mosquitoes were head-squashed and examined by IF. Detection of viral antigen in the head tissues of the recipient mosquitoes was interpreted as a transmission of dengue virus.

I. Titration of Dengue Viruses by Immunofluorescence in Microtiter Plates

A fast, reliable, and inexpensive method was developed for titration of dengue viruses in microtiter plates with an indirect fluorescent-antibody technique (Schoepp and Beaty, 1984). Briefly, 8 or 16 replicates of each serial 10-fold dilution of infectious virus suspensions (mosquitoes or cell cultures) were inoculated into 96-well, flat-bottom microtiter plates (0.05 ml per well). Each well was seeded with 0.15 ml of a suspension of Ae. albopictus C6/36 cells containing 10^6 cells per ml. The plates were kept in a humid incubator at 27°C for 7 days. After incubation, the cell culture fluid was aspirated from each well with a 14-gauge, 1.5 inch needle attached to a vacuum system. To fix the cells, cold buffered acetone was added to each well and the plates were incubated for 30 minutes. After fixation, the buffered acetone was aspirated from each well. The plates were air dried and stained immediately or stored at -20°C until staining and examination for viral antigen by indirect fluorescent-antibody technique. To determine the sensitivity of the microtiter assay, duplicate titrations of the samples were done in Lab-Tek slides as described above. Statistical comparisons of the two methods were done.

J. Pathogenesis

To determine precise anatomic location of vaccine virus, selected mosquitoes were dissected and organ systems were examined by IF for the presence of viral antigen (Beaty and Thompson, 1976, 1978).

K. Reversion

To determine if the attenuated dengue vaccine strains reverted to virulence after mosquito passage, the virus was passaged 3 times each in Ae. albopictus and Ae. aegypti mosquitoes. Samples of the passaged virus were sent to WRAIR where each was analyzed for plaque morphology and temperature sensitivity.

L. Interference Between Dengue Viruses in Aedes albopictus Mosquitoes

In dengue virus endemic areas multiple dengue types often circulate at the same time, making it possible for the mosquito or man to become dually of multiply infected. In the mosquito, co-infection with taxonomically different viruses can result in either interference between the viruses or independent replication. To determine if interference occurs during dual infection with dengue viruses, Ae. albopictus mosquitoes were intrathorocically inoculated initially with either dengue-1, dengue-2, or dengue-4 small plague, temperature sensitive candidate vaccine viruses. At 7 or 14 days post-inoculation, groups of mosquitoes were challenged with homologous parental strains of dengue virus types 1, 2, and 4 or with heterologous viruses (Table 8). The heterologous challenge viruses were a related flavivirus, West Nile and an unrelated bunyavirus, LaCrosse. The mosquitoes were maintained at 27°C for 14 days after challenge and then stored at -70°C.

To determine which viruses replicated in the mosquitoes, individual mosquitoes were triturated, diluted (1:10), and inoculated into 96-well microtiter plates seeded with Ae. albopictus C6/36 cells as described above.

of five was stained with one of five specific monoclonal antibodies using the indirect fluorescent antibody technique (Schoepp and Beaty, 1984). In addition, the virus in mosquitoes was phenotyped. Duplicate aliquots of triturated mosquitoes were sent to WRAIR where each was titered in LLC-MK_2 cells at 35°C and 38.5°C to determine temperature sensitivity and plaque morphology.

V. Summary of Studies

A. Development of Techniques

1. Efficient Oral Infection of Mosquitoes

During the granting period, considerable time and effort was committed in developing an efficient technique to orally infect Ae. aegypti and Ae. albopictus mosquitoes. High titered virus stocks were prepared in mosquitoes, LLC-MK_2, J-111 and BHK-21 vertebrate cell lines and the C6/36 clone of Igarashi's Ae. albopictus cell line. Frozen virus stocks were thawed and mixed with 20% sucrose in either defibrinated blood or washed human red blood cells. Even with these high titered virus-blood preparations, only a small percentage of engorging mosquitoes became infected.

To determine if Fc receptors might be a determinate of virus infection of midgut cells, blood meals were prepared with different concentrations of homologous and heterologous antibody and cells mixed with a thawed virus preparation grown in LLC-MK_2 cells. Again the results were not encouraging; few mosquitoes became infected.

To see if serum factors might mediate midgut infection, a crude approximation of viremic human blood was prepared. Whole human blood (with yellow fever antibodies) was mixed with a minimal amount of heparin and added to a flask of C6/36 cells which had been previously (7 days) infected with PR-159 virus. A control flask contained infected C6/36 cells only. Four days later the cells (rbc's, leukocytes and C6/36 cells) were mechanically removed and fed to mosquitoes. Washed rbc's were added to the control flask and fed to mosquitoes. After an extrinsic incubation period of 14 days, the mosquitoes were examined for the presence of viral antigen. Over 20% of the mosquitoes were infected. Subsequently the technique was modified to that described in the previous section. Mosquito oral infection rates of up to 100% (Table 1) were achieved, depending upon the virus titer of the cell culture preparation. Infection rates were consistently higher than those obtained with frozen virus stocks.

Several hypotheses could account for the success of the unfrozen virus meal preparations: 1) freezing virus preparations might somehow alter glycoprotein conformation rendering the virus less capable of interacting with midgut cell receptors, 2) virus in cells might be protected from proteolytic enzymes or other toxic substances, 3) cell or membrane associated virus might better interact with midgut cells. A pilot study was conducted to test these hypotheses. Parent dengue-2 virus was used to infect C6/36 cells. After 7 days incubation, cells were scraped from the flask and separated by centrifugation (800xg, 30 minutes). The infected cells and supernatant were split into 2 treatment groups, one was held at room temperature while the other was frozen and thawed 3 times. These 4 preparations were mixed with human red blood cells and 10% sucrose in serum and fed to Ae. albopictus mosquitoes. Infection rates (Table 2) ranged from 100% for mosquitoes ingesting unfrozen virus-infected cells to 72% for mosquitoes ingesting frozen supernatant virus. These initial results are difficult to interpret: 1) the centrifugation regimen was inadequate to remove membrane associated virus from the "cell-free" supernatant and 2) quick-freezing and thawing is probably not analogous to long term storage of

virus stocks at -70°C. Nonetheless, in each comparison, the unfrozen virus preparation resulted in higher infection and subsequent dissemination rates than the frozen virus preparations. To determine the extent of these observations, similar studies were conducted with LaCrosse (LAC) virus, family bunyaviridae, and its vector, Ae. triseriatus. Mosquitoes were fed meals of washed human rbc's, 10% sucrose in calf serum and 1) a frozen infected mouse brain suspension, 2) infected cell culture supernatant, or 3) infected BHK-21 cells. A fourth infectious source was viremic suckling mice. Virus titers ranged from 5.5 to 6.8 $\log_{10}$ $TCID_{50}$/ml (Table 3). Engorged mosquitoes were examined by IF for viral antigen 14 days post-feeding.

Fifty percent of the mosquitoes that fed on a viremic mouse circulating 5.5 $\log_{10}TCID_{50}$/ml of LAC virus became infected (Table 3). In contrast, of those mosquitoes ingesting a meal containing 6.0 to 6.5 $\log_{10}TCID_{50}$/ml of a frozen mouse brain virus preparation, only 3% (1/38) became infected. Those mosquitoes engorging an artificial meal containing either cell culture supernatant virus or infected cells had 30% (12/40) and 49% (17/35) infection rates respectively. Interestingly those mosquitoes ingesting the unfrozen virus preparations had higher disseminated infection rates than those mosquitoes feeding on the viremic mouse.

2. Improved In vitro Oral Transmission Assay

Studies were conducted to determine if the oil in vitro transmission assay could be successfully applied to detect dengue virus transmission.

Mosquitoes were inoculated with either dengue-2 parent or vaccine virus, yellow fever virus, or LaCrosse virus. After 1 week incubation, wings and legs were removed from the mosquitoes and the probosci were inserted into capillary pipettes charged with 3.5 ul of Cargille immersion oil. After 30-60 minutes exposure, mosquitoes were removed and examined by IF for the presence of viral antigen. Charged capillaries containing the mosquito saliva were placed in microcentrifuge tubes containing 0.1 ml of 20% FBS in phosphate buffered saline. The tubes were centrifuged twice for 1 minute in order to force the contents of the capillary into the diluent. Centrifuge tubes were then frozen. To assay for virus transmission, the contents of the tubes were inoculated into recipient mosquitoes. After 14 days, recipient mosquitoes were head squashed and processed by IF. Detection of antigen indicated virus transmission.

To compare the in vitro technique to in vivo transmission, sibling mosquitoes, infected with either yellow fever or LaCrosse virus (because dengue viruses typically do not cause morbidity or mortality in mice), were separated into two groups. One group of each was permitted to engorge upon suckling mice; the other was assayed for transmission using the in vitro technique.

Transmission of both yellow fever and LaCrosse virus was demonstrated. In these pilot studies (Tables 4 and 5), some difficulties were encountered with mouse and recipient mosquito survival. Nonetheless, the results were encouraging. Interestingly, after 1 week extrinsic incubation, one mosquito without detectable yellow fever viral antigen in the head squash preparation transmitted virus (Table 4). Thus it seems that the assay can detect transmission before sufficient viral antigen to detect by IF accumulates in

the head tissues. Since the mosquitoes were inoculated parenterally, all were presumably infected. In those instances where the *in vivo* and *in vitro* techniques could be compared, yellow fever transmission rates were similar.

Similar results were obtained with LaCrosse virus transmission attempts (Table 5). After 2 weeks incubation, 15/16 (94%) mosquitoes that could be assayed using the *in vitro* transmission technique were demonstrated to have transmitted. Of the mosquitoes that could be assayed using the *in vivo* technique, 12/14 (86%) transmitted.

On the basis of these results, a pilot study using intrathoracically inoculated mosquitoes was conducted to compare the transmission rates of the parent and vaccine dengue-2 viruses using the *in vitro* technique. Results are shown in Table 6. Transmission of both viruses was detected. Interestingly, the mosquitoes inoculated with the parent virus received approximately 0.8 $\log_{10}$ $TCID_{50}$; whereas the mosquitoes infected with the vaccine virus received approximately 2.6 logs. Nonetheless, after 1 week extrinsic incubation, transmission rates by parent and vaccine infected mosquitoes were similar. The total transmission rate for mosquitoes infected with the parent virus was 87% (14/16) and 75% (9/12) for mosquitoes infected with vaccine virus.

The use of the improved oil *in vitro* assay permitted testing of mosquitoes in a fraction of the time necessary using the old *in vitro* assay.

3. Titration of Dengue Viruses by Immunofluorescence in Microtiter Plates

Virus titers obtained by the microtiter plate system were comparable with those obtained by the multichambered (Lab-Tek) slide system (Table 7). There was no statistically significant differences ($P<0.05$) between the titers for particular samples by either method. It should also be noted that the standard errors of titers obtained by the microtiter system were generally markedly lower than those obtained in the chambered-slide system. This difference was due to the use of 8 or 16 replicates of each sample in microtiter plates as compared with only 4 in multichambered slides.

4. Interference Between Dengue Viruses in Aedes albopictus Mosquitoes

To determine if interference to dengue superinfection occurs in dengue infected vectors, mosquitoes were initially infected with small plaque, temperature sensitive candidate vaccine viruses and then challenged with parent strains (Table 8). Thus if replication of the dengue vaccine virus interfered with that of the challenge virus, the recovered virus would be expected to be temperature sensitive and small plaque morphology.

In Tables 9-14, the phenotypes of virus recovered from mosquitoes initially inoculated with dengue vaccine viruses and challenged with wild-type viruses are presented. After 7 or 14 days post-inoculation, the temperature sensitivity data, represented by efficiency of plaquing (EOP = titer at the non-permissive temperature 38.5°C/titer at the permissive temperature 35°C) did not result in a clear differentiation of mosquitoes infected with vaccine virus and those with mixed infections of wild-type dengue virus. At the non-permissive temperature of 38.5°C, many of the *ts* vaccine viruses were leaky and continued to grow almost as efficiently as the parent viruses. Plaque morphology allowed us to better analyze the

interference phenomenon (Tables 9-14), but conclusions concerning the extent of interference to superinfection were difficult to make.

To better understand the interference phenomenon, specific monoclonal antibodies to the 5 viruses were used in an indirect fluorescent antibody test to unequivocally identify viruses present in the mosquitoes. The results of these experiments are seen in Tables 15-20.

When dengue-1 infected mosquitoes were challenged at 7 days (Table 15) with dengue types 2 and 4, interference was not detected. Dengue-1 virus and each dengue challenge virus infected the mosquitoes and replicated. In addition, there was no interference between dengue-1 virus and WN or LAC.

When challenged at 14 days PI (Table 16), there was still no demonstratable interference between any of the viruses. The dengue-1 infected mosquitoes challenged with dengue-4 were infected with both viruses. Upon reexamination of the plates we found mosquitoes challenged at 7 days had about 50-75% of the cells infected with dengue-4, but when challenged at 14 days, approximately 10% of the cells were infected with dengue-4. The molecular basis of this phenomenon remains to be determined. However, it may suggest that interference in the mosquito is time dependent and that dengue-1 infections may render mosquitoes resistant to superinfection after extended extrinsic incubation periods.

When dengue-2 infected mosquitoes were challenged at 7 or 14 days with the same viruses, there was no demonstratable interference between dengue-2 and dengue-1 (Tables 17 and 18). However, challenge with dengue-4 virus did not result in superinfection. Therefore, interference between these two viruses had occurred. Challenge at 14 days resulted in the same situation (Table 18).

When dengue-4 infected mosquitoes were challenged after 7 days with dengue-1 and 2, it was expected that dengue-4 and dengue-1 would replicate independently of each other while dengue-4 would interfere with dengue-2 (Table 19). Dengue-4 and dengue-1 did not interfere with each other. In addition, when mosquitoes infected with dengue-4 were challenged with dengue-2, superinfection did not occur (Table 19).

When challenged after 14 days extrinsic incubation, there was no interference between dengue-4 virus and dengue-1; both replicated independently in the mosquito (Table 20). Challenge of dengue-4 infected mosquitoes with dengue-2 resulted in the majority of the mosquitoes exhibiting no interference. However, 2 mosquitoes were only infected with dengue-4 indicating that at least in these mosquitoes interference to superinfection occurred.

From this limited study, it would seem that interference between the dengue viruses in mosquitoes depends on the viruses. Clearly more work is required to make any conclusions about the interference phenomenon in mosquitoes. However, it is apparent that what occurs <u>in vivo</u> in the mosquito may differ from that which occurs <u>in vitro</u>. Dittmar et al. (1982), using monoclonal antibody probes, determined that mosquito cells infected with one dengue virus type for at least 20 hours, were resistant to superinfection with other dengue virus types.

B. Summary of Dengue-1 Studies

1. Dengue-1 Candidate Vaccine - TP-56

Viruses: Dengue-1 parent (P7)
Dengue-1 candidate vaccine (TP-56)

Status: No longer considered a candidate vaccine

Threshold of Infection

To determine the comparative threshold of oral infection for the two viruses, Ae. aegypti mosquitoes were permitted to engorge upon meals containing serial 10-fold dilutions of virus preparations. After 14 days extrinsic incubation at 27°C, mosquitoes were processed by IF for the presence of viral antigen. The parent meal titered 8.0 $\log_{10}TCID_{50}$ per ml, and the vaccine meal titered 7.8 logs. The Ae. aegypti mosquitoes only became infected when they engorged the meals prepared from the undiluted virus preparations. The disseminated infection rates were 30% (14/46) for mosquitoes engorging the parent virus and 39% (12/31) for those engorging the vaccine virus (Table 21). The rates did not differ statistically. The low infection rates were surprising. After ingestion of considerably less dengue-1 vaccine virus (Table 22), 42% (10/24) of Ae. albopictus mosquitoes contained detectable virus after 7 days extrinsic incubation.

Growth Curves

To determine the comparative ability of the dengue-1 viruses to replicate in Ae. albopictus, mosquitoes were permitted to engorge blood meals containing 7.5 $\log_{10}TCID_{50}$ per ml of the parent virus or 7.8 logs of the vaccine virus. At selected days post-feeding, mosquitoes were harvested and stored at -70°C. Mosquitoes were subsequently triturated and titrated in Ae. albopictus C6/36 cells. Mosquito titers are shown in Table 23. To calculate geometric mean titers, mosquitoes with only trace amounts of virus were assigned a titer of 1 $\log_{10}TCID_{50}$. Mosquito titrations without endpoints were assigned values equal to the most conservative calculated titer.

The TP-56 vaccine apparently replicated more rapidly in the mosquitoes than the parent virus (Table 23). There was an "eclipse" phase for the parent virus; on days 2, 4, 6, 8, post-infection there was no demonstrable virus in the mosquitoes. In contrast, all mosquitoes tested contained demonstrable vaccine virus on days 4, 6, and 8; and geometric mean titers were 2.9 $\log_{10}TCID_{50}$, 4.1, and 4.0, respectively. By days 16 and 19 post-infection, geometric mean titers of parent and vaccine infected mosquitoes were not significantly different.

Oral Transmission

To determine oral transmission capability, saliva samples were collected from Ae. albopictus mosquitoes orally infected with parent or vaccine virus. The parent blood meal titered 7.5 $\log_{10}TCID_{50}$ per ml, and the vaccine meal titered 7.8 logs. After 21 days extrinsic incubation at 27°C, mosquitoes from which saliva was collected were processed by

immunofluorescence for the presence of viral antigen. Infection and dissemination rates were 80% (16/20) for parent virus and 100% (20/20) for vaccine virus. However transmission rates for parent and vaccine viruses were 13% (2/16) and 5% (1/19), respectively (Table 24).

Pathogenesis

Pathogenesis experiments were completed and samples are available for analysis.

Reversion

Reversion experiments were completed and samples are available for analysis.

Summary

The dengue-1 (non-mutagenized) candidate vaccine virus was not altered in its ability to infect and to replicate in Ae. aegypti or Ae. albopictus mosquitoes. Oral infection rates with the vaccine virus were invariably as high or higher for the parent virus. Further, the vaccine virus replicated more efficiently in mosquitoes after oral infection than the parent virus. During these studies, we were informed by Dr. Kenneth H. Eckels that this particular candidate vaccine was phenotypically different in cell culture than other candidate vaccines and would not be used in clinical trials. Thus we did not complete the pathogenesis and reversion studies.

Nonetheless, on the basis of the oral infection and replication trials, this candidate vaccine would not seem to be sufficiently attenuated to preclude mosquito infection during engorgement on viremic vaccinees.

2. Dengue-1 Candidate Vaccine - 45AZ5

Viruses: Dengue-1 parent (P8D6)
Dengue-1 vaccine (45AZ5)

Status: No longer considered a candidate vaccine

Threshold of Infection

To determine the comparative ability of the parent and vaccine viruses to infect Ae. albopictus and Ae. aegypti, mosquitoes were permitted to engorge blood meals containing serial 10-fold dilutions of each virus. After 21 days extrinsic incubation at 27°C the mosquitoes were stored at -70°C and are available for analysis.

Growth Curves

To determine the comparative ability of the parent and vaccine dengue-1 viruses to infect and replicate in both Ae. albopictus and Ae. aegypti, mosquitoes were allowed to engorge blood meals of known titer and were extrinsically incubated at 27°C or 33°C. On selected days post-infection, mosquitoes engorging parent and vaccine virus were frozen at -70°C until analysis. The parent and vaccine virus blood meals ingested by Ae. albopictus mosquitos titered 7.5 and 7.3 $\log_{10}$ $TCID_{50}$ per ml, respectively (Tables 25 and 26). Ae. albopictus mosquitoes ingesting either parent or vaccine virus and incubated at 27°C exhibited an eclipse period initially (Table 25). Virus was first detected on day 7 post-feeding. Parent virus titers increased to approximately 3.0-3.5 logs, while vaccine titers increase to a slightly higher level of approximately 4.0 logs.

Data is incomplete for the treatment group of mosquitoes incubated at 33°C, but it appears that at least parent virus replicates faster, eliminating the eclipse period seen in the 27°C treatment group (Table 26).

Parent and vaccine blood meals on which Ae. aegypti mosquitoes engorged titered 7.8 and 8.0 logs respectively (Tables 27 and 28). Due to contamination, the early course of parent-virus replication could not be followed in the 27°C incubation group (Table 27). However after day 5 post-feeding, growth of parent virus was similar to that which occurred in Ae. albopictus mosquitoes, increasing to a mean titer of approximately 3.5 logs. The vaccine virus infected Ae. aegypti mosquitoes less efficiently than the parent virus (Table 27). After day 5 post feeding 96% (23/24) of the mosquitoes engorging parent virus were infected compared to only 50% (14/28) of the mosquitoes engorging vaccine.

Ae. aegypti mosquitoes incubated at 33°C appeared to be less efficiently infected with both parent and vaccine virus than mosquitoes incubated at 27°C (Table 28).

Oral Transmission

To compare the ability of Ae. albopictus mosquitoes to transmit parent and vaccine virus, saliva samples were collected from orally infected mosquitoes. Partial data from mosquitoes extrinsically incubated for 14

disseminated, and were transmitted by this mosquito species (Table 29) Mosquitoes extrinsically incubated at 27°C transmitted parent and vaccine virus 50% (3/6) and 67% (4/6) of the time. When incubated at 33°C parent and vaccine transmission rates were 50% (3/6) and 38% (3/8), respectively. Additional samples, which do not appear in this summary are available for analysis.

Pathogenesis

Pathogenesis experiments were completed and samples are available for analysis.

Reversion

Reversion experiments were completed and samples are available for analysis.

Summary

The dengue-1 attenuated candidate vaccine virus (45AZ5) was similar to the parent virus in its ability to infect, and to replicate in Ae. aegypti or Ae. albopictus mosquitoes. Oral infection rates with the vaccine virus were as high or higher when compared to the parent virus. During these studies we were informed by Dr. Kenneth H. Eckels that this candidate vaccine was no longer considered a candidate vaccine. Thus, pathogenesis and reversion studies were not completed.

Nonetheless, on the basis of oral infection, replication, and transmission data this candidate vaccine would not seem to be sufficiently attenuated to preclude mosquito infection during engorgement on viremic vaccinees.

C. Summary of Dengue-2 Studies

1. Dengue-2 Candidate Vaccine - PR-159/S-1

Viruses: Dengue-2 parent (PR-159)
Dengue-2 candidate vaccine (PR-159/S-1)

Status: Work completed.

Threshold of Infection

The results of the comparative oral infection experiments are presented in Tables 30 and 31. Dengue viruses grew to higher titers in C6/36 than in LLC-MK_2 cells. When Ae. aegypti mosquitoes ingested the parent virus grown in C6/36 cells at titers ranging from 4.2 to 8.2 $\log_{10}MID_{50}$/ml, 75% (145/194) became infected; 97% (141/145) of the infected mosquitoes developed a disseminated infection (Table 30). In contrast when mosquitoes fed on the same titer of vaccine virus grown in C6/36 cells, 21% (39/183) became infected; 59% (23/39) of the infected mosquitoes developed a disseminated infection. The overall rate of virus dissemination to mosquito head tissues was 72% (141/194) for the parent virus and 12% (23/183) for the vaccine virus. When the infectious titer of virus grown in C6/36 cells was 5.2-6.2 $\log_{10}MID_{50}$/ml, 67% (35/52) of the mosquitoes exposed to the parent virus became infected in contrast to 6% (4/60) exposed to the vaccine virus. The mosquito 50% oral infectious dose $(OID)_{50}$/ml for the parent virus was between 5.4 and 5.7 $\log_{10}MID_{50}$ and $\geq$7.2 $\log_{10}MID_{50}$/ml for the attenuated vaccine virus.

Similar results were obtained with parent and vaccine viruses grown in LLC-MK_2 cells (Table 31). Overall infection rates were obtained by combining the results obtained using virus stocks prepared in C6/36 cells with those obtained using virus stocks grown in LLC-MK_2 cells (Table 31). The total infection rate for mosquitoes ingesting bloodmeals containing 3.7 to 8.2 $\log_{10}MID_{50}$ per ml of the parent virus was 56% (220/396); in contrast, only 16% (66/397) of those ingesting the same amount of the vaccine virus became infected.

Growth Curves

Mosquitoes were permitted to engorge blood meals containing approximately 7.2 $\log_{10}$ $TCID_{50}$ per ml of either the parent or the vaccine dengue-2 virus. On days 0, 3, 5, 7, 9, 11, and 14 post-feeding, 5 females that had engorged on the parent and 5 females that had engorged on the vaccine virus were frozen at -70°C and subsequently titered for virus content by mosquito inoculation.

The virus growth curves for orally infected Ae. aegypti mosquitoes are presented in Figure 1. Titration of 5 mosquitoes immediately after exposure to blood meals containing 7.2 $\log_{10}TCID_{50}$/ml of virus resulted in a geometric mean titer of 4.7 $\log_{10}TCID_{50}$/ml for the parent PR-159 virus and 5.0 $\log_{10}TCID_{50}$/ml for the attenuated S-1 virus. Titers fell on day 3 post-feeding and increased to day 7. Thirty mosquitoes were fed on the respective virus strains and titrated on days 3 to 14 post-feeding. Of those that engorged the meal containing the parent virus, 27 (90%) became infected; 18 (60%) engorging the vaccine virus became infected. In general, the parent

the vaccine strain (Figure 1).

Oral Transmission

Mosquitoes were allowed to engorge on infectious blood meals containing approximately 7.2 $\log_{10}TCID_{50}$/ml of either parent or attenuated dengue-2 virus (Table 31). All (22/22) of the Ae. aegypti feeding on the parent virus became infected and developed disseminated infections by 21 days extrinsic incubation. Fifty-five percent (16/29) of the mosquitoes engorging on the attenuated virus blood meal became infected, but only 28% (8/29) developed a disseminated infection. Fourteen percent (3/22) of the mosquitoes infected with the parent strain transmitted virus to a serum-sucrose drop. None of the mosquitoes infected with the vaccine strain transmitted.

Pathogenesis

A number of mosquitoes infected with the vaccine virus were dissected in order to ascertain which tissues/organs were involved in virus replication. In many cases, viral antigen was found in large amounts in the mesenteral tissues only. The fore and hindguts as well as ovaries, ventral nerve chord, salivary glands and fat body were free of demonstrable S-1 viral antigen. It would appear that although virus was replicating in the midgut, it was unable to mature and escape into the hemocoel or unable to attach and replicate in secondary organ systems. The molecular basis for this attenuation is not known.

Reversion

Studies were conducted to determine if the S-1 vaccine virus would revert to virulence during mosquito passage. Two biological markers, plaque size and temperature sensitivity were used originally to characterize the attenuated virus. The S-1 clone produced small plaques and did not grow at temperatures of 39°C or higher. These markers were used to address the possibility that the S-1 virus might revert to virulence (large plaque size and growth at 39°C) after passage in mosquitoes. The dengue-2 viruses were characterized in the infectious blood meal and after growth in orally infected mosquitoes (Table 33). The S-1 cloned virus remained temperature sensitive when grown in C6/36 cells or LLC-MK_2 cells and after passage in mosquitoes. Plaque sizes were heterogeneous, although no large plaques were seen. Surprisingly the parent virus apparently became attenuated (temperature sensitive) after passage in the C6/36 cells, and the attenuation seemed to be accentuated by passage in the mosquito vector.

To determine the comparative susceptibility of the two main vector species of dengue-2, Ae. aegypti and Ae. albopictus mosquitoes were permitted to engorge upon serial 10-fold dilutions of the parent and vaccine viruses (Table 34). After 14 days extrinsic incubation, mosquitoes were examined by IF for the presence of viral antigen.

Ae. albopictus mosquitoes seemed to be more susceptible than Ae. aegypti to oral infection by both the parent and vaccine viruses. Parent virus antigen was detected in 97% (68/70) and 66% (46/68), respectively, of the Ae. albopictus and Ae. aegypti that engorged the parent virus. Vaccine

the Ae. albopictus and Ae. aegypti that engorged the parent virus. Vaccine virus antigen was detected in 65% (40/65) and 20% (18/88) respectively of the Ae. albopictus and Ae. aegypti that engorged the vaccine virus (Table 34).

Summary

The S-1 vaccine strain seemed to be markedly less efficient than the parent PR-159 strain in interactions with potential vector species. The S-1 vaccine was considerably less efficient in oral infection of vectors (Tables 30, 31,and 34); it was considerably less efficient in developing disseminated infection (Tables 30 and 31); when disseminated infection did occur, it was later than that for the PR-159 strain; and finally the vaccine strain was less efficiently transmitted (Table 32).

Thus, the dengue-2, S-1 vaccine virus, which is attenuated for man and animals, was also modified in its ability to infect orally and to be transmitted by Ae. aegypti mosquitoes. Oral infection only occurred in a substantial number of mosquitoes when the infectious titer of the meal was relatively high (Tables 30, 31, and 34). None of the Ae. aegypti mosquitoes orally infected with the vaccine virus subsequently transmitted. It seems reasonable to speculate that the virus infection in those mosquitoes that fed on the vaccines was restricted to the midgut.

The parent dengue-2 virus was transmitted by infected Ae. aegypti mosquitoes; the S-1 vaccine was not. However, the numbers were not sufficient to draw conclusions. The Ae. albopictus mosquitoes seemed to be more vector competent (oral infection) than the Ae. aegypti mosquitoes (Table 34).

The attenuated virus remained temperature sensitive after replication in mosquitoes. The plaque morphology was not uniformly small, although large plaques characteristic of the parent virus were not detected. Temperature sensitivity and plaque size/morphology are biological markers which may or may not be related or correlated with the parameters of vector competency.

D. Summary of Dengue-3 Studies

1. Dengue-3 candidate vaccine - CH53489, Clone 24/28

Viruses: Dengue-3 parent (CH53489)
Dengue-3 vaccine (CH53489, Clone 24/28)

Status: Work completed.

Threshold of Infection

To determine the comparative threshold of oral infection for the two viruses, both Ae. albopictus and Ae. aegypti mosquitoes were permitted to engorge serial 10-fold dilutions of blood meal preparations. After 14 day extrinsic incubation at 27°C, mosquitoes were processed by IF for the presence of viral antigen. The parent and vaccine meals on which Ae. albopictus mosquitoes engorged titered 8.4 and 8.2 $\log_{10}TCID_{50}$ per ml respectively. Infection and dissemination rates are shown in Table 35. Parent and vaccine virus both appeared equally efficient in oral infection of mosquitoes. Parent and vaccine overall infection rates were 94% (189/201) and 92% (149/162). Parent virus disseminating to head tissues in 84% (160/191) of the mosquitoes was slightly more efficient than vaccine virus disseminating 74% (117/158) of the time. The median oral infectious dose (OID_{50}) for both viruses were <5.0 logs.

The parent and vaccine meals on which Ae. aegypti mosquitoes engorged titered 7.8 logs per ml each. Infection and dissemination rates are shown in Table 36. As with Ae. albopictus mosquitoes, parent and vaccine virus were equally efficient in oral infection of mosquitoes with overall infection rates of 91% (90/99) and 89% (71/80) respectively. In addition the dissemination rates of both viruses indicate that parent and vaccine virus were equally efficient, with rates of 89% (88/99) and 85% (68/80) respectively. The OID_{50} for both viruses were <4.8 logs.

Growth Curves

To determine the comparative ability of the parent and vaccine dengue-3 viruses to infect and to replicate in Ae. albopictus and Ae. aegypti, mosquitoes were permitted to engorge high titered infectious blood meal preparations and extrinsically incubated at 27°C or 33°C. On selected days post-feeding mosquitoes engorging parent and vaccine virus were frozen at -70°C. Subsequently mosquitoes were triturated and titrated using IF.

The parent and vaccine blood meals that Ae. albopictus mosquitoes engorged titered 7.8 and 8.3 logs respectively. Mosquitoes extrinsically incubated at 27°C or 33°C had no detectable eclipse period (Tables 37 and 38). Initially mosquitoes engorging the respective blood meals titered ≥5.4 logs, after 3 days post-feeding the titers dipped to about 3 logs and then started to increase. Mosquitoes engorging parent and vaccine virus and incubated at 27°C increased to about 4.0 logs and 3.5 logs respectively. Mosquitoes incubated at 33°C reached higher titers, between 5.0-5.5 logs, indicating that dengue-3 virus grows more efficiently at a higher extrinsic incubation temperature.

The parent and vaccine blood meals that Ae. aegypti mosquitoes engorged

titered 6.0 $\log_{10}TCID_{50}$ per ml each. The growth curves of dengue-3 virus in this species of mosquito were similar to those seen with Ae. albopictus (Table 39 and 40). Again there was no detectable eclipse period. Parent and vaccine infected mosquitoes incubated at 27°C had virus titers of approximately 5.0 logs each. Parent and vaccine infected mosquitoes incubated at 33°C contained slightly lower titers of virus, approximately 4.0-4.5 logs. Interestingly, peaks in both parent and vaccine virus growth curves were reached between days 7 and 14 post-feeding and then decreased about 1 log by day 21.

Oral Transmission

To determine oral transmission capability, saliva samples were collected from Ae. albopictus and Ae. aegypti mosquitoes orally infected with parent or virus blood meals. After 14 and 21 days extrinsic incubation mosquitoes donating saliva were processed by immunofluorescence for the presence of viral antigen. Infection, and dissemination, rates were remarkably similar for the two viruses. (Tables 41 and 42). Interestingly, neither parent nor vaccine virus were readily transmitted by the mosquitoes.

Pathogenesis

Selected mosquitoes engorging either parent or vaccine virus were dissected on days 14 and 21 post-feeding and major organ systems were examined by immunofluorescence for evidence of infection. However, the mosquitoes examined were also induced to feed on oil charged capillary tubes which resulted in the oil masking viral antigen. This masking effect made determination of pathogenesis impossible.

Reversion

Studies were conducted to determine if clone 24/28 vaccine virus would revert to virulence during mosquito passage. Two biological markers, plaque size and temperature sensitivity were used originally to characterize the attenuated virus. After 3 mosquito passages the vaccine virus remained temperature sensitive and of small plaque morphology (Table 43).

Summary

Dengue-3, CH53489, clone 24/28 vaccine virus is not modified in its ability to orally infect either Ae. albopictus or Ae. aegypti mosquitoes. However, neither parent or vaccine viruses were efficiently transmitted despite high dissemination rates of the virus to head tissues, and presumably to the salivary glands. Whether this phenomenon may be due to a problem with the in vitro transmission assay for dengue-3 virus is being determined.

Comparative oral infection studies of each species indicated that the dengue-3 viruses are extremely efficient at infecting and replicating in mosquitoes.

The higher incubation temperature of 33°C did result in increased peak infectious titers in Ae. albopictus mosquitoes. In Ae. aegypti mosquitoes the peak titers declined with time. The molecular basis of the decline in titer is unknown.

The attenuated virus remained temperature sensitive after replication in mosquitoes. This is not surprising since the mosquitoes were maintained at temperatures well below 38.5°C. The plaque morphology was uniformly small and large plaques characteristic of the parent virus strain were not detected. Temperature sensitivity and plaque morphology are biological markers that may or may not be related or correlated with the parameters of vector competency.

E. Summary of Dengue-4 Studies

1. Dengue-4 Candidate Vaccine - H-241, Lot 1

Viruses: Dengue-4 parent (H-241)
Dengue-4 vaccine (H-241, Lot 1)

Status: No longer considered a candidate vaccine.

Threshold of Infection

To determine the comparative ability of the parent and vaccine viruses to infect Ae. albopictus, mosquitoes were permitted to engorge blood meals containing sequential dilutions of the respective blood meals (Table 44). After 21 days extrinsic incubation at 27°C, mosquitoes were processed by immunofluorescence for the presence of viral antigen. Infection and dissemination rates are shown in Table 44. The parent virus was markedly more efficient in oral infection of Aedes albopictus. The infection rates of mosquitoes ingesting > 6.0 $\log_{10}TCID_{50}$ of the parent virus was 72% (70/97). In contrast, only 15% (16/105) of the mosquitoes ingesting >6.0 $\log_{10}TCID_{50}$ of the vaccine virus became infected. The oral infectious $dose_{50}$ (OID_{50}) for the parent virus was approximately 5.5 $\log_{10}$ $TCID_{50}$ per ml; the OID_{50} for the vaccine was > 7.0 logs.

Growth Curves

To determine the comparative ability of the parent and vaccine dengue-4 viruses to infect and to replicate in Ae. albopictus, three separate studies have been conducted. In all three studies, mosquitoes engorged high titered infectious blood meals. On selected days post-infection, mosquitoes engorging the parent and the vaccine virus were frozen at -70°C. Subsequently, mosquitoes were triturated and titrated. In two of the studies, mosquitoes were randomly separated after engorgement and held at different ambient temperatures for the extrinsic incubation period.

In the first trial, the parent meal titered 9.5 $\log_{10}TCID_{50}$ per ml. The vaccine meal titered 7.0 logs. The ambient temperature for the extrinsic incubation period was 27°C. By day 5 post-infection 3 of 4 mosquitoes tested were positive; the geometric mean titer was 3.1 logs (Table 45). Vaccine virus was not detected in a substantial proportion of mosquitoes until 16 days post-infection; the geometric mean titer was 2.1 logs. The geometric mean titer for the mosquitoes infected with the parent virus was 4.7 logs on day 16. However, because of the large discrepancy between parent and vaccine virus meal titers (9.5 vs. 7.0) comparison of results in this trial is of questionable importance.

In the second trial, both the parent and the vaccine virus meals titered 7.0 $\log_{10}$ $TCID_{50}$ per ml. After engorgement, mosquitoes engorging either the parent or vaccine virus were randomly separated into two groups; one group of each was incubated at 27°C, the other at 30°C. Titers of individual mosquitoes incubated at 27°C are shown in Table 46, those incubated at 30°C in Table 47. Geometric mean titers of mosquitoes infected with the respective viruses at each extrinsic incubation are shown in Table 48.

The parent virus efficiently infected and replicated in mosquitoes. After 5 days extrinsic incubation at 27°C, 75% (3/4) mosquitoes contained significant quantities of parent virus. In contrast, the first mosquito containing a significant amount of vaccine virus was not detected until 9 days extrinsic incubation (Table 46). Overall infection rates for mosquitoes processed between 5 and 21 days extrinsic incubation were 86% (24/28) for those engorging the parent virus and 16% (5/32) for those engorging the vaccine virus.

The same phenomenom was noted when the mosquitoes were incubated at 30°C (Table 47). There was no discernable eclipse phase for those mosquitoes ingesting the parent dengue-4 virus; all contained significant titers of virus by 3 days extrinsic incubation. In contrast, the first mosquito that contained a significant amount of vaccine virus was not detected until 9 days extrinsic incubation. It is noteworthy that on day 11, all 4 mosquitoes titrated contained significant amounts of virus (Table 47). This may be due to a sampling error; the mosquitoes were probably inadvertently obtained from a parent virus cage. These mosquitoes were not included in further analyses. The overall infection rates for mosquitoes incubated at 30°C and processed between 3 and 21 days extrinsic incubation were 81% (26/32) for those engorging the parent virus and 10% (3/28) for those engorging the vaccine virus (Table 47).

Incubation of mosquitoes at 30°C tended to result in faster replication of the parent virus. Mosquitoes that were incubated at 30°C contained significant quantities of virus by 3 days post-infection; those incubated at 27°C required 2 additional days extrinsic incubation to develop significant virus titers (Tables 46-48).

In the third trial, parent and vaccine virus blood meals titered 9.0 and 8.5 $\log_{10}TCID_{50}$ per ml respectively. Mosquitoes engorging parent virus and incubated at 27°C had no eclipse period (Table 49). The titers of the mosquitoes increased rapidly and leveled off at approximately 4.5 $\log_{10}TCID_{50}$ per ml after 5 days post-feeding. In contrast mosquitoes engorging vaccine virus had an eclipse period until virus was first detected on day 7. Mosquitoes did not become consistently infected and no real pattern could be discerned.

Mosquitoes that engorged the same blood meals as above but extrinsically incubated at 33°C showed similar patterns (Table 50). Mosquitoes engorging parent virus had no eclipse period and titers increased rapidly to a slightly higher mean of approximately 5.0 logs. Again mosquitoes ingesting vaccine virus were not consistently infected.

Infection Rates at Different Temperatures

Infection rates were determined for mosquitoes incubated at 27°C or 30°C (Table 51). Mosquitoes ingested a meal of either the parent or vaccine virus that titered 7.0 $\log_{10}TCID_{50}$ per ml. Of the mosquitoes ingesting the parent virus, 77% (30/39) became infected when incubated at 27°C; 78% (21/27) became infected at 30°C. Of those mosquitoes ingesting the vaccine virus, 19% (8/42) became infected at 27°C incubation temperature (Table 51). Interestingly, only one of the mosquitoes incubated at 30°C became infected.

Oral Transmission

Saliva samples have been collected from mosquitoes on selected days post-feeding and are stored at -70°C. Partial data has been compiled and appears in Table 52.

Pathogenesis

In trial 2, selected mosquitoes engorging either parent or vaccine virus were dissected and major organ systems examined by immunofluorescence for evidence of infection. None of the mosquitoes ingesting the vaccine virus (titer - 7.0 $\log_{10}TCID_{50}$ per ml) contained detectable antigen. In contrast with the parent virus, 3 of 5 mosquitoes incubated at 27°C and 4 of 5 incubated at 33°C contained detectable antigen (Table 53). For the positive mosquitoes incubated at 33°C, all organ systems contained 3-4+ levels of fluorescence (Table 53). Only one of the mosquitoes incubated at 27°C contained a large quantity of antigen in secondary target organs. The other two had demonstrable antigen in the headsquash preparation but not in the salivary glands, ovariole sheaths, or abdominal ganglia.

Reversion

Reversion experiments have been completed and samples are available for analysis.

Summary

The dengue-4 candidate vaccine virus was markedly less efficient than its parent virus in infection and replication in Ae. albopictus mosquitoes. The OID_{50} of the vaccine virus was >7.0 $\log_{10}TCID_{50}$ per ml (Table 44). In fact, only 24% (7/29) mosquitoes became infected after engorging a meal containing 7.0 logs of vaccine virus. The OID_{50} for the parent virus was approximately 5.5 logs. Thus it would require more than 100 times as much vaccine virus as parent to obtained the similar infection rates. After infection of mosquitoes vaccine virus replicated at a slower rate and to a lower titer than the parent virus (Tables 45-51). Mosquitoes engorging higher titered meals of parent virus tended to reach maximum virus titers faster (Tables 49 and 50) as did increasing the extrinsic incubation temperature (Tables 47,50, and 51). In addition the maximum titers reached were also higher. Increased blood meal titers and incubation temperatures did not appear to affect infection and replication of vaccine virus in mosquitoes.

Oral transmission trials were not completed once the dengue-4 vaccine virus was no longer considered a candidate (Table 57) but on the basis of the marked attenuation of the vaccine virus for vector infection and replication, this candidate would seem to be sufficiently attenuated to preclude mosquito infection by engorgement on recent vaccines. Furthermore, even in the event that mosquito infection did occur, subsequent transmission of the virus would seem unlikely because of reduced ability of the vaccine to replicate in mosquitoes.

2. Dengue-4 Candidate Vaccine - SG D6

Viruses: Dengue-4 parent (H-241)
Dengue-4 vaccine (SG D6)

Status: Repeated attempts to grow virus stocks in C6/36 cells were unsuccessful. Since this virus did not grow in C6/36 cells, we could not analyze it in our system.

VI. Publications

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VII. Tables and Figures

Table 1. Infection and transmission rates for *Aedes albopictus* (OAHU) orally infected with dengue-2 parent (PR-159) and vaccine (S-1) viruses.[a]

	Dengue-2 virus[b]	
	Parent	Vaccine
No. mosquitoes exposed	20	29
No. infected (%)	20 (100)	16 (55)
No. transmitting[c] (%)	15 (75)	9 (56)

[a] Extrinsic incubation period was 21-24 days.

[b] Each blood meal contained 7.3 $\log_{10}TCID_{50}$/ml post-feeding.

[c] *In vitro* transmission technique of Aitken (1977).

Table 2. Infection rates for Aedes albopictus (OAHU) ingesting dengue-2 (PR-159) virus.[a]

Unfrozen				Frozen			
Cell associated virus		Supernatant virus		Cell associated virus		Supernatant virus	
Infected (%)	Disseminated (%)	Infected (%)	Disseminated (%)	Infected (%)	Disseminated (%)	Infected (%)	Disseminated[d] (%)
21/21[c] (100)	16/21 (76)	29/30 (97)	25/30 (83)	26/31 (84)	19/31 (61)	21/29 (72)	11/29 (38)

[a] Meals consisted of 1 ml virus preparation grown in Aedes albopictus C6/36 cells, 1 ml washed human rbc and 0.5 ml 10% sucrose in calf serum.

[b] Frozen and thawed three times before use.

[c] No. positive over no. tested by IF.

[d] Viral antigen detected in head tissues.

Table 3. Infection rates of *Aedes triseriatus* mosquitoes fed on various La Crosse virus preparations.

Meal[b]	Titer[c]	Infection rate (%)[a] Gut	Dissemination[e]
Frozen virus[d]	6.0-6.5	1/38 (3)	1/38 (3)
Cell culture supernatant	5.5-6.3	12/40 (30)	8/40 (20)
Cells and supernatant	6.0-6.8	17/35 (49)	10/35 (29)
Viremic suckling mice	5.5	8/16 (50)	1/16 (6)

[a] No. positive over no. tested for viral antigen by FA after 14 days extrinsic incubation.

[b] Meals consisted of virus preparations, washed human red blood cells, calf serum and sucrose.

[c] $Log_{10}TCID_{50}/ml$.

[d] Suckling mouse brain preparation; also used to infect BHK-21 cells and inoculate suckling mice for viremic meal.

[e] Virus antigen detected by FA in head tissues.

Table 4. Comparison of an *in vitro* technique and engorgement upon suckling mice for an assay of yellow fever virus transmission by mosquitoes.

	In vitro				Suckling mice			
	Donor incubation period				Donor incubation period			
	1 week		2 weeks		1 week		2 weeks	
No.	Donor	Recep.	Donor	Recip.	Donor	Mouse death	Donor	Mouse death
1	+[a]	+	+	+	+	+	+	NF[c]
2	-	-	+	NS[b]	+	-	+	+
3	+	+	+	NS	+	+	+	NF
4	+	NS	+	NS	-	-	+	+
5	+	+	+	NS	+	-	+	NF
6	+	+	+	NS	+	+	+	NF
7	+	NS	+	NS	+	-	+	NF
8	-	+	+	+	+	-	+	NF
9	+	+	-	-	+	+	-	-
10	+	+	-	-	+	+	-	-

[a] Results of IF examination of headsquash preparations.

[b] None of the recipients survived the two-week incubation period.

[c] Mosquito did not feed on mouse.

Table 5. Comparison of an *in vitro* technique and engorgement upon suckling mice for an assay of LaCrosse virus transmission by mosquitoes.

	In vitro				Suckling mice			
	Donor incubation period				Donor incubation period			
	1 week		2 weeks		1 week		2 weeks	
No.	Donor	Recep.	Donor	Recip.	Donor	Mouse death	Donor	Mouse death
1	+[a]	+	+	+	+	D[c]	+	NF[d]
2	+	+	+	+	+	D	+	+
3	+	+	+	+	+	D	+	NF
4	+	NS[b]	+	+	-	D	+	NF
5	+	NS	+	+	+	D	-	NF
6	+	+	+	+	+	D	+	NF
7	+	+	+	+	+	D	+	-
8	+	+	+	-	+	D	+	-
9	+	NS	+	+	+	D	+	+
10	+	NS	+	+	+	D	+	+

[a] Results of IF examination of headsquash preparations.

[b] None of the recipients survived the one-week incubation period.

[c] Non-virus associated mouse death.

[d] Mosquito did not feed on mouse.

Table 6. *In vitro* transmission of dengue-2 parent (PR-159) and vaccine (S-1) viruses using the oil capillary technique.

	Parent				Vaccine			
	Donor incubation period				Donor incubation period			
	1 week		2 weeks		1 week		2 weeks	
No.	Donor	Recep.	Donor	Recip.	Donor	Mouse death	Donor	Mouse death
1	+[a]	+	+	+	+	+	+	-
2	+	-	+	-	+	NS	+	+
3	+	+	+	+	+	NS	+	+
4	-	+	+	+	+	-	+	NS
5	-	NS[b]	+	+	+	-	+	+
6	+	NS	+	+	+	NS	+	NS
7	-	NS	+	+	+	+	+	NS
8	+	NS	+	+	+	+	+	+
9	+	+	+	+	+	NS	+	+
10	+	+	-	+	+	NS	+	+

[a] Results of IF examination of headsquash preparations.

[b] None of the recipients survived the two-week incubation period.

Table 7. Comparisons of virus titers of dengue viruses in microtiter plates multi-chambered slides.[a]

Virus Sample	Virus Titer	
	Microtiter plates	Chambered slides
Working Stock Virus		
Den-1 parent (Western Pacific, 1974)	6.55±0.15	6.00±0.25
Den-1 vaccine (45AZ5)	6.93±0.12	6.50±0.24
Den-2 parent (PR-159)	6.55±0.15	6.50±0.31
Den-2 vaccine (PR-159/S-1)	8.36±0.14	8.75±0.22
Den-3 parent (CH53489)	7.05±0.11	6.75±0.22
Den-3 vaccine (Clone 24/28)	6.49±0.15	6.25±0.33
Den-4 parent (H-241)	6.43±0.17	6.25±0.22
Den-4 vaccine (H-241, Lot 1)	4.68±0.12	5.00±0.25
Blood Meal Preparations		
Experiment 84002		
Den-1 parent (P8 D6)	7.68±0.23	7.75±0.22
Den-1 vaccine (45AZ5)	7.68±0.19	7.50±0.31
Experiment 84004		
Den-1 parent (Western Pacific, 1974)	6.80±0.17	6.75±0.22
Den-1 vaccine (45AZ5)	7.23±0.15	7.50±0.31
Mosquito Suspensions		
Den-1 vaccine (45AZ5)		
Aedes aegypti (Day 0, 27°C)[c]		
Mosquito 1	5.30±0.20	5.00±0.25
2	4.80±0.00[d]	3.50±0.00[d]
3	3.80±0.00	3.50±0.00
4	5.13±0.19	4.50±0.31
Den-4 vaccine (H-241, Lot 1)		
Aedes albopictus (Day 21, 33°C)		
Mosquito 1	0	0
2	0	0
3	4.44±0.19	4.50±0.23
4	0	0
Den-1 parent (Western Pacific, 1974)		
Aedes aegypti (Day 21, 33°C)		
Mosquito 1	4.36±0.12	4.75±0.22
2	4.05±0.11	4.25±0.22
3	5.05±0.23	4.50±0.31
4	3.80±0.17	4.00±0.25

[a] No statistically significant difference was found ($P<0.05$) between the two methods.

[b] Titers are expressed as $\log_{10}$ (50% tissue culture infective dose) per milliliter ± standard error.

[c] Day of extrinsic incubation and ambient temperature and given in parentheses after the specific epithet of each test group of four (designated 1 through 4) mosquitoes.

[d] Estimates of standard error cannot be calculated by the Karber method.

Table 8. Viruses and titers of each inoculated into *Aedes albopictus* mosquitoes

VIRUSES	TITER[1]
INITIAL VIRUSES	
DENGUE-1 VACCINE (45AZ5)	4.8
DENGUE-2 VACCINE (PR 159/S-1)	4.2
DENGUE-4 VACCINE (H-241, LOT 1)	4.5
CHALLENGE VIRUSES	
DENGUE-1 PARENT (P-7)	5.0
DENGUE-2 PARENT (PR-159)	4.5
DENGUE-4 PARENT (H-241)	4.5
WEST NILE (EG 101)	4.4
LA CROSSE	4.5

[1] $LOG_{10}TCID_{50}$ per ml

Table 9. Virus phenotype after replication in *Aedes albopictus* mosquitoes initially inoculated with dengue-1 vaccine and challenged 7 days post inoculation

INOCULATED VIRUSES		RECOVERED VIRUS TITERS[1]		PLAQUE	
DAY 0	DAY 7	35°C	38.5°C	MORPHOLOGY	EOP[2]
CONTROL	CONTROL	<0.70	N.D.[3]	-	-
DENGUE-1 VACCINE	CONTROL	4.51	3.07	(SMALL)	0.04
CONTROL	DENGUE-1 PARENT	4.24	3.53	(SMALL)	0.13
DENGUE-1 VACCINE	DENGUE-1 PARENT	4.80	3.57	SMALL	0.06
CONTROL	DENGUE-2 PARENT	2.97	<1.15	MIXED	<0.04
DENGUE-1 VACCINE	DENGUE-2 PARENT	4.68	3.31	MIXED	0.04
CONTROL	DENGUE-4 PARENT	3.28	2.85	MIXED	0.37
DENGUE-1 VACCINE	DENGUE-4 PARENT	4.75	3.44	(SMALL)	0.05
CONTROL	WEST NILE	6.09	5.95	MIXED	0.81
DENGUE-1 VACCINE	WEST NILE	5.23	5.10	MIXED	0.77
CONTROL	LA CROSSE	4.28	3.49	MIXED	0.17
DENGUE-1 VACCINE	LA CROSSE	4.48	3.23	MIXED	0.06

1 $LOG_{10}TCID_{50}$ per ml
2 Efficiency of Plaquing = Titer 38.5°C/Titer 35°C
3 Not Determined

Table 10. Virus phenotype after replication in Aedes albopictus mosquitoes initially inoculated with dengue-2 vaccine and challenged 7 days post-inoculation

INOCULATED VIRUSES		RECOVERED VIRUS TITERS[1]		PLAQUE	
DAY 0	DAY 7	35°C	38.5°C	MORPHOLOGY	EOP[2]
CONTROL	CONTROL	<0.70	N.D.[3]	-	-
DENGUE-2 VACCINE	CONTROL	5.65	5.16	MIXED	0.37
CONTROL	DENGUE-1 PARENT	4.24	3.53	(SMALL)	0.13
DENGUE-2 VACCINE	DENGUE-1 PARENT	5.65	5.07	MIXED	0.32
CONTROL	DENGUE-2 PARENT	2.97	<1.15	MIXED	<0.04
DENGUE-2 VACCINE	DENGUE-2 PARENT	5.22	3.63	MIXED	0.03
CONTROL	DENGUE-4 PARENT	3.28	2.85	MIXED	0.37
DENGUE-2 VACCINE	DENGUE-4 PARENT	5.13	3.60	MIXED	0.03
CONTROL	WEST NILE	6.09	5.95	MIXED	0.81
DENGUE-2 VACCINE	WEST NILE	5.94	5.73	MIXED	0.61
CONTROL	LA CROSSE	4.28	3.49	MIXED	0.17
DENGUE-2 VACCINE	LA CROSSE	4.93	3.58	MIXED	0.09

1 $LOG_{10}TCID_{50}$ per ml
2 Efficiency of Plaquing = Titer 38.5°C/Titer 35°C
3 Not Determined

Table 11. Virus phenotype after replication in Aedes albopictus mosquitoes initially inoculated with dengue-4 vaccine and challenged 7 days post-inoculation

INOCULATED VIRUSES		RECOVERED VIRUS TITERS[1]		PLAQUE	
DAY 0	DAY 7	35°C	38.5°C	MORPHOLOGY	ROP[2]
CONTROL	CONTROL	<0.70	N.D.[3]	-	-
DENGUE-4 VACCINE	CONTROL	2.88	<0.70	SMALL	<0.01
CONTROL	DENGUE-1 PARENT	4.24	3.53	(SMALL)	0.13
DENGUE-4 VACCINE	DENGUE-1 PARENT	4.60	3.94	MIXED	0.21
CONTROL	DENGUE-2 PARENT	2.97	<1.15	MIXED	<0.04
DENGUE-4 VACCINE	DENGUE-2 PARENT	3.31	<0.70	SMALL	<0.004
CONTROL	DENGUE-4 PARENT	3.28	2.85	MIXED	0.37
DENGUE-4 VACCINE	DENGUE-4 PARENT	4.31	2.89	SMALL	0.14
CONTROL	WEST NILE	6.09	5.95	MIXED	0.81
DENGUE-4 VACCINE	WEST NILE	6.28	6.24	MIXED	0.92
CONTROL	LA CROSSE	4.28	3.49	MIXED	0.17
DENGUE-4 VACCINE	LA CROSSE	4.45	4.39	MIXED	0.91

1 $LOG_{10}TCID_{50}$ per ml
2 Efficiency of Plaquing = Titer 38.5°C/Titer 35°C
3 Not Determined

Table 12. Virus phenotype after replication in *Aedes albopictus* mosquitoes initially inoculated with dengue-1 vaccine and challenged 14 days post-inoculation

INOCULATED VIRUSES		RECOVERED VIRUS TITERS[1]		PLAQUE	
DAY 0	DAY 14	35°C	38.5°C	MORPHOLOGY	EOP[2]
CONTROL	CONTROL	<0.70	N.D.[3]	-	-
DENGUE-1 VACCINE	CONTROL	4.70	3.90	SMALL	0.18
CONTROL	DENGUE-1 PARENT	4.37	3.43	(SMALL)	0.11
DENGUE-1 VACCINE	DENGUE-1 PARENT	4.49	<1.28	SMALL	<0.0005
CONTROL	DENGUE-2 PARENT	3.37	<0.90	MIXED	<0.005
DENGUE-1 VACCINE	DENGUE-2 PARENT	4.69	3.57	SMALL	0.08
CONTROL	DENGUE-4 PARENT	3.92	3.29	MIXED	0.37
DENGUE-1 VACCINE	DENGUE-4 PARENT	4.75	2.03	SMALL	0.002
CONTROL	WEST NILE	5.70	5.51	MIXED	0.61
DENGUE-1 VACCINE	WEST NILE	5.49	5.34	MIXED	0.84
CONTROL	LA CROSSE	4.05	3.23	MIXED	0.18
DENGUE-1 VACCINE	LA CROSSE	4.36	3.08	MIXED	0.05

1 $LOG_{10}TCID_{50}$ per ml
2 Efficiency of Plaquing = Titer 38.5°C/Titer 35°C
3 Not Determined

Table 13. Virus phenotype after Rreplication in Aedes albopictus mosquitoes initially inoculated with dengue-2 vaccine and challenged 14 days post-inoculation

INOCULATED VIRUSES		RECOVERED VIRUS TITERS[1]		PLAQUE	
DAY 0	DAY 14	35°C	38.5°C	MORPHOLOGY	EOP[2]
CONTROL	CONTROL	<0.70	N.D.[3]	-	-
DENGUE-2 VACCINE	CONTROL	N.D.	N.D.	N.D.	N.D.
CONTROL	DENGUE-1 PARENT	4.37	3.43	(SMALL)	0.11
DENGUE-2 VACCINE	DENGUE-1 PARENT	5.36	4.66	MIXED	0.21
CONTROL	DENGUE-2 PARENT	3.37	<0.90	MIXED	<0.005
DENGUE-2 VACCINE	DENGUE-2 PARENT	5.44	4.62	MIXED	0.15
CONTROL	DENGUE-4 PARENT	3.92	3.29	MIXED	0.37
DENGUE-2 VACCINE	DENGUE-4 PARENT	5.40	5.13	MIXED	0.54
CONTROL	WEST NILE	5.70	5.51	MIXED	0.61
DENGUE-2 VACCINE	WEST NILE	5.94	5.76	MIXED	0.61
CONTROL	LA CROSSE	4.05	3.23	MIXED	0.18
DENGUE-2 VACCINE	LA CROSSE	5.36	4.41	MIXED	0.11

1 $LOG_{10}TCID_{50}$ per ml

2 Efficiency of Plaquing = Titer 38.5 °C/Titer 35°C

3 Not D.

Table 14. Virus phenotype after replication in Aedes albopictus mosquitoes initially inoculated with dengue-4 vaccine and challenged 14 days post-inoculation

INOCULATED VIRUSES		RECOVERED VIRUS TITERS[1]		PLAQUE	
DAY 0	DAY 14	35°C	38.5°C	MORPHOLOGY	EOP[2]
CONTROL	CONTROL	<0.70	N.D.[3]	-	-
DENGUE-4 VACCINE	CONTROL	2.76	<0.70	SMALL	<0.01
CONTROL	DENGUE-1 PARENT	4.37	3.43	(SMALL)	0.11
DENGUE-4 VACCINE	DENGUE-1 PARENT	4.06	2.89	SMALL	0.09
CONTROL	DENGUE-2 PARENT	3.37	<0.90	MIXED	<0.005
DENGUE-4 VACCINE	DENGUE-2 PARENT	3.21	<1.70	SMALL	<0.004
CONTROL	DENGUE-4 PARENT	3.92	3.29	MIXED	0.37
DENGUE-4 VACCINE	DENGUE-4 PARENT	N.D.	N.D.	N.D.	N.D.
CONTROL	WEST NILE	5.70	5.51	MIXED	0.61
DENGUE-4 VACCINE	WEST NILE	6.02	5.89	MIXED	0.68
CONTROL	LA CROSSE	4.05	3.23	MIXED	0.18
DENGUE-4 VACCINE	LA CROSSE	4.25	3.46	MIXED	0.15

1 $LOG_{10}TCID_{50}$ per ml
2 Efficiency of Plaquing = Titer 38.5°C/Titer 35°C
3 Not Determined

Table 15. Monoclonal detection of virus replication in Aedes albopictus mosquitoes initially inoculated with dengue-1 and challenged 7 days post-inoculation

INOCULATED VIRUSES		MONOCLONAL REACTIVITY				
INITIAL	CHALLENGE	DENGUE-1	DENGUE-2	DENGUE-4	WEST NILE	LA CROSSE
DENGUE-1	CONTROL	+	-	-	-	-
DENGUE-1	DENGUE-2	+	+	-	-	-
DENGUE-1	DENGUE-4	+	-	+	-	-
DENGUE-1	WEST NILE	+	-	-	+	-
DENGUE-1	LA CROSSE	+	-	-	-	+
CONTROLS						
	DENGUE-1	+	-	-	-	-
	DENGUE-2	-	+	-	-	-
	DENGUE-4	-	-	+	-	-
	WEST NILE	-	-	-	+	-
	LA CROSSE	-	-	-	-	+

Table 16. Monoclonal detection of virus replication in *Aedes albopictus* mosquitoes initially inoculated with dengue-2 and challenged 7 days post-inoculation

INOCULATED VIRUSES		MONOCLONAL REACTIVITY				
INITIAL	CHALLENGE	DENGUE-1	DENGUE-2	DENGUE-4	WEST NILE	LA CROSSE
DENGUE-2	CONTROL	-	+	-	-	-
DENGUE-2	DENGUE-1	+	+	-	-	-
DENGUE-2	DENGUE-4	-	+	-	-	-
DENGUE-2	WEST NILE	-	+	-	+	-
DENGUE-2	LA CROSSE	-	+	-	-	+
CONTROLS						
	DENGUE-1	+	-	-	-	-
	DENGUE-2	-	+	-	-	-
	DENGUE-4	-	-	+	-	-
	WEST NILE	-	-	-	+	-
	LA CROSSE	-	-	-	-	+

Table 17. Monoclonal detection of virus replication in Aedes albopictus mosquitoes initially inoculated with dengue-4 and challenged 7 days post-inoculation

INOCULATED VIRUSES		MONOCLONAL REACTIVITY				
INITIAL	CHALLENGE	DENGUE-1	DENGUE-2	DENGUE-4	WEST NILE	LA CROSSE
DENGUE-4	CONTROL	-	-	+	-	-
DENGUE-4	DENGUE-1	+	-	+	-	-
DENGUE-4	DENGUE-2	-	+	+	-	-
DENGUE-4	WEST NILE	-	-	+	+	-
DENGUE-4	LA CROSSE	-	-	+	-	+
CONTROLS						
	DENGUE-1	+	-	-	-	-
	DENGUE-2	-	+	-	-	-
	DENGUE-4	-	-	+	-	-
	WEST NILE	-	-	-	+	-
	LA CROSSE	-	-	-	-	+

Table 18. Monoclonal detection of virus replication in Aedes albopictus mosquitoes initially inoculated with dengue-1 and challenged 14 days post-inoculation

INOCULATED VIRUSES		MONOCLONAL REACTIVITY				
INITIAL	CHALLENGE	DENGUE-1	DENGUE-2	DENGUE-4	WEST NILE	LA CROSSE
DENGUE-1	CONTROL	+	-	-	-	-
DENGUE-1	DENGUE-2	+	+	-	-	-
DENGUE-1	DENGUE-4	+	-	+	-	-
DENGUE-1	WEST NILE	+	-	-	+	-
DENGUE-1	LA CROSSE	+	-	-	-	+
CONTROLS						
DENGUE-1		+	-	-	-	-
DENGUE-2		-	+	-	-	-
DENGUE-4		-	-	+	-	-
WEST NILE		-	-	-	+	-
LA CROSSE		-	-	-	-	+

Table 19. Monoclonal detection of virus replication in Aedes albopictus mosquitoes initially inoculated with dengue-2 and challenged 14 days post-inoculation

INOCULATED VIRUSES		MONOCLONAL REACTIVITY				
INITIAL	CHALLENGE	DENGUE-1	DENGUE-2	DENGUE-4	WEST NILE	LA CROSSE
DENGUE-2	CONTROL	-	+	-	-	-
DENGUE-2	DENGUE-1	+	+	-	-	-
DENGUE-2	DENGUE-4	-	+	-	-	-
DENGUE-2	WEST NILE	-	+	-	+	-
DENGUE-2	LA CROSSE	-	+	-	-	+
CONTROLS						
DENGUE-1		+	-	-	-	-
DENGUE-2		-	+	-	-	-
DENGUE-4		-	-	+	-	-
WEST NILE		-	-	-	+	-
LA CROSSE		-	-	-	-	+

Table 20. Monoclonal detection of virus replication in Aedes albopictus mosquitoes initially inoculated with dengue-4 and challenged 14 days post-inoculation

INOCULATED VIRUSES		MONOCLONAL REACTIVITY				
INITIAL	CHALLENGE	DENGUE-1	DENGUE-2	DENGUE-4	WEST NILE	LA CROSSE
DENGUE-4	CONTROL	-	-	+	-	-
DENGUE-4	DENGUE-1	+	-	+	-	-
DENGUE-4	DENGUE-2	-	±*	+	-	-
DENGUE-4	WEST NILE	-	-	+	+	-
DENGUE-4	LA CROSSE	-	-	+	-	+
	CONTROLS					
	DENGUE-1	+	-	-	-	-
	DENGUE-2	-	+	-	-	-
	DENGUE-4	-	-	+	-	-
	WEST NILE	-	-	-	+	-
	LA CROSSE	-	-	-	-	+

* 3 Mosquitoes Positive per 5 Mosquitoes Tested

Table 21. Infection rates of Aedes aegypti mosquitoes orally infected with graded doses of dengue-1 parent (P7) or attenuated vaccine (TP-56) viruses grown in C6/36 cells.[a]

Dilution of Blood Meal[b]	Infected[c] (%)	Disseminated[d] (%)	Infected (%)	Disseminated (%)
10^{0}	14/46 (30)	NT[e]	12/31 (39)	NT
10^{-1}	2/10 (20)	0/10 (0)	3/10 (30)	2/10 (20)
10^{-2}	1/10 (10)	0/10 (0)	2/10 (20)	0/10 (0)
10^{-3}	0/10 (0)	0/10 (0)	1/10 (10)	0/10 (0)
Total	17/76 (22)	0/30 (0)	18/61 (30)	2/30 (7)

[a] Mosquitoes were maintained at 27°C during extrinsic incubation.

[b] Parent virus titer 8.0 $\log_{10}TCID_{50}$ per ml (MID_{50}) vaccine virus titer 7.8 MID_{50}.

[c] Number mosquitoes positive for dengue-1 viral antigen in midgut/number tested.

[d] Number mosquitoes positive for dengue-1 viral antigen in head tissues/ number tested.

[e] NT = not tested.

Table 22. Replication of dengue-1 vaccine virus (TP-56) after oral infection of *Aedes albopictus* mosquitoes.

	Titer of virus ($TCID_{50}$)												
	Days post infection												
Mosquito	0	1	2	3	4	5	6	7	8	9	10	11	14
1	2.3	-	<2.0	<1.0	-[a]	-	<1.0	2.0[b]	2.8	3.3	-	5.0	-
2	2.8	-	-	<1.0	-	-	-	-	2.0	3.8	-	-	3.5
3	2.5	-	-	-	-	-	-	-	-	3.3	-	-	4.0
4	3.0	-	-	<1.0	<1.0	-	-	-	-	-	-	-	-

[a] No virus detected in titration.

[b] $Log_{10}TCID_{50}$ per mosquito.

Table 23. Replication of dengue-1 parent (P7) and vaccine (TP-56) viruses in *Aedes albopictus* mosquitoes after oral infection[a]

		Titer of virus ($TCID_{50}$)										
		Days Post-Feeding										
Virus[a]	Mosquito	0	2	4	6	8	9	10	12	14	16	19
Parent	1	3.3	0	0	0	0	0	3.3	3.5	4.8	≥4.8	5.5
	2	3.5	0	0	0	0	4.0	≥5.5	4.5	2.8	4.8	5.5
	3	3.3	0	0	0	0	2.8	0	≥5.3	4.5	5.0	≥5.3
	4	3.3	0	0	0	0	0	0	NT[b]	6.0	5.8	≥5.3
Mean Titer		3.4	0	0	0	0	1.7	≥2.2	≥4.4	4.5	5.1	≥5.4
Vaccine	1	5.0	0	2.8	2.8	4.5	2.5	4.0	4.5	6.8	≥5.5	≥5.5
	2	4.5	0	2.5	4.8	3.5	3.3	≥5.5	5.5	4.8	≥5.5	≥5.5
	3	4.8	0	3.0	4.3	4.3	5.0	4.5	6.0	5.8	5.5	≥5.5
	4	4.5	TR[c]	3.3	4.5	3.8	TR	4.5	6.0	5.5	5.5	≥5.5
Mean Titer		4.7	0.3	2.9	4.1	4.0	3.0	≥4.6	5.5	5.7	≥5.5	≥5.5

[a] Dengue-1 parent virus bloodmeal titered 7.5 $\log_{10}TCID_{50}$ per ml.
Dengue-1 vaccine virus bloodmeal titered 7.8 $\log_{10}TCID_{50}$ per ml.

[b] NT = Not tested

[c] TR = Trace

Table 24. Infection, dissemination, and transmission rates of *Aedes albopictus* mosquitoes engorging a blood meal of dengue-1 parent (P7) or vaccine (TP-56) virus.[a]

	Parent	Vaccine
Infection	16/20 (80)[b]	20/20 (100)
Dissemination	16/20 (80)	20/20 (100)
Transmission[c]	2/16 (13)	1/19 (5)

[a] Blood meal titers: Parent virus 7.5 $\log_{10}TCID_{50}$/ml
Vaccine virus 7.3 $\log_{10}TCID_{50}$/ml

[b] Days extrinsic incubation post-infection.

[c] Number positive/total (%)

Table 25. Replication of dengue-1 parent and vaccine (45AZ5) viruses in *Aedes albopictus* mosquitoes after oral infection and extrinsic incubation at 27°C.

		Days Post-Feeding[a]									
Virus	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	3.0	0	0	0	0	2.5	2.5	ND[b]	ND	3.8
	2	3.8	0	0	0	0	2.5	3.5	ND	ND	3.0
	3	3.3	0	0	2.3	0	3.0	2.8	ND	ND	3.0
	4	3.5	0	0	3.0	2.0	2.0	3.3	ND	ND	4.3
Mean		3.4	0	0	1.3	0.5	2.5	3.0	-	-	3.5
Vaccine	1	4.8	0	0	0	3.3	0	5.0	4.3	4.5	3.0
	2	5.0	0	0	2.8	0	3.0	4.5	4.5	4.8	2.0
	3	4.8	0	0	2.5	2.0	2.5	3.0	4.0	5.0	4.5
	4	4.8	0	0	0	2.8	4.8	4.3	2.8	5.0	4.8
Mean		4.9	0	0	1.3	2.0	2.6	4.2	3.9	4.8	3.6

[a] Blood meal titers: Parent virus 7.5 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 7.3 $\log_{10}$ $TCID_{50}$ per ml

[b] ND = Not Done

Table 26. Replication of dengue-1 parent and vaccine (45AZ5) viruses in Aedes albopictus mosquitoes after oral infection and extrinsic incubation at 33°C.

		Days Post-Feeding[a]									
Virus	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	3.0	3.0	3.5	0	≥5.5	-[b]	-	-	-	-
	2	3.8	3.8	2.5	4.3	≥5.5	-	-	-	-	-
	3	3.3	≥5.5	3.3	2.8	4.5	-	-	-	-	-
	4	3.5	2.0	2.8	4.5	≥5.5	-	-	-	-	-
Mean		3.4	≥3.6	3.0	3.9	≥5.3	-	-	-	-	-
Vaccine	1	4.8	-	-	-	-	-	-	-	NDc	-
	2	5.0	-	-	-	-	-	-	-	ND	-
	3	4.8	-	-	-	-	-	-	-	ND	-
	4	4.8	-	-	-	-	-	-	-	ND	-
Mean		4.9	-	-	-	-	-	-	-	-	-

[a] Blood meal titers: Parent virus 7.5 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 7.3 $\log_{10}$ $TCID_{50}$ per ml

[b] Mosquitoes available for analysis

[c] ND = Not Done

Table 27. Replication of dengue-1 parent and vaccine (45AZ5) viruses in Aedes aegypti mosquitoes after oral infection and extrinsic incubation at 27°C.

		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	4.0	ctm[c]	ctm	Tr[b]	4.2	4.3	2.8	ctm	4.0	3.0
	2	4.8	ctm	ctm	Tr	ctm	4.5	4.5	4.5	3.5	2.8
	3	4.5	ctm	ctm	0	Tr	3.5	Tr	4.3	3.5	0
	4	4.5	0	ctm	3.5	ctm	4.3	ctm	3.5	4.0	3.8
Mean		4.5	-	-	1.8	(2.6)	4.2	(2.8)	(4.1)	3.8	3.2
Vaccine	1	5.0	0	0	2.8	0	0	2.8	3.8	4.3	0
	2	3.5	0	0	0	0	2.5	0	4.3	0	0
	3	3.5	0	0	0	0	3.5	3.5	4.0	3.8	3.8
	4	4.5	0	0	2.8	0	0	3.3	3.3	0	0
Mean		4.1	0	0	1.4	0	1.5	(3.2)	3.9	(4.1)	(3.8)

[a] Blood meal titers: Parent virus 7.8 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.0 $\log_{10}$ $TCID_{50}$ per ml

[b] Tr = Trace

[c] ctm = contaminated

Table 28. Replication of dengue-1 parent and vaccine (45AZ5) viruses in Aedes aegypti mosquitoes after oral infection and extrinsic incubation at 33°C.

		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	4.0	0	2.8	0	0	ctm[b]	4.5	ctm	ctm	4.8
	2	4.8	0	0	0	3.3	2.0	ctm	Tr[c]	ctm	4.3
	3	4.5	3.3	3.8	3.5	3.0	3.5	3.8	ctm	2.5	4.5
	4	4.5	0	0	0	0	2.5	4.0	ctm	4.3	4.0
Mean		4.5	0.8	1.7	0.9	1.6	(2.7)	(4.1)	-	(3.4)	4.4
Vaccine	1	5.0	0	2.8	0	0	0	3.5	0	ND[d]	ctm
	2	3.5	0	0	0	0	0	ctm	3.3	ND	ctm
	3	3.5	0	0	2.2	0	0	0	4.0	ND	0
	4	4.5	Tr	2.5	2.8	4.0	0	0	4.3	ND	4.3
Mean		4.1	0.3	1.3	1.3	1.0	0	(1.2)	3.9	-	(2.2)

[a] Blood meal titers: Parent virus 7.8 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.0 $\log_{10}$ $TCID_{50}$ per ml

[b] ctm = contaminated

[c] Tr = Trace

[d] ND = Not Done

Table 29. Infection, dissemination, and transmission rates of Aedes albopictus mosquitoes engorging a blood meal of dengue-1 parent or vaccine (45AZ5) virus[a] and extrinsically incubated at 27°C or 33°C.

		Parent		Vaccine	
		7 days[b]	14 days	7 days	14 days
27°C	Infection	3/15 (20)[c]	11/19 (58)	5/15 (33)	7/20 (8)
	Dissemination	0/15 (0)	6/19 (32)	2/15 (13)	10/20 (50)
	Transmission	ND[d]	3/6 (50)	ND	4/6 (67)
33°C	Infection	10/15 (67)	14/20 (70)	12/15 (80)	14/20 (70)
	Dissemination	2/15 (13)	13/20 (65)	3/15 (20)	12/20 (60)
	Transmission	ND	3/6 (50)	ND	3/8 (38)

[a] Blood meal titers: Parent virus 7.5 $\log_{10}TCID_{50}$/ml
Vaccine virus 7.3 $\log_{10}TCID_{50}$/ml

[b] Days extrinsic incubation post-infection.

[c] Number positive/total (%)

[d] ND = not done.

Table 30. Infection rates for Aedes aegypti orally infected with graded doses of dengue-2 parent (PR-159) and attenuated (S-1) viruses grown in $LLC\text{-}MK_2$ cells.

Titer of bloodmeal[a]	Parent virus (PR-159)		Vaccine virus (S-1)	
	Infected[b] (%)	Disseminated[c] (%)	Infected (%)	Disseminated (%)
8.2 - 7.2	55/56 (98)	55/56 (98)	8/8 (100)	8/8 (100)
7.2 - 6.2	41/42 (98)	41/42 (98)	25/55 (45)	11/55 (20)
6.2 - 5.2	35.52 (67)	35/52 (67)	4/60 (7)	3/60 (5)
5.2 - 4.2	14/44 (32)	10/44 (23)	2/60 (3)	1/60 (2)
Total	145/194 (75)	141/194 (73)	39/183 (21)	23/183 (13)

[a] Log_{10} mosquito infective dose 50 per ml

[b] Number mosquitoes positive for dengue-2 viral antigen in midgut/number tested.

[c] Number mosquitoes positive for dengue-2 viral antigen in head tissues/number tested.

Table 31. Infection rates for Aedes aegypti orally infected with graded doses of dengue-2 parent (PR-159) and attenuated (S-1) viruses grown in LLC-MK_2 cells.

Titer of bloodmeal[a]	Parent virus (PR-159)		Vaccine virus (S-1)	
	Infected[b] (%)	Disseminated[c] (%)	Infected (%)	Disseminated (%)
7.2 - 6.7	36/49 (73)	32/49 (65)	20/60 (33)	8/60 (13)
6.7 - 5.7	30/50 (60)	26/50 (52)	6/68 (9)	3/68 (4)
5.7 - 4.7	8/59 (14)	5/59 (8)	1/42 (2)	1/42 (2)
4.7 - 3.7	1/44 (2)	1/44 (2)	0/44 (0)	0/44 (0)
Total	75/202 (37)	64/202 (32)	27/214 (13)	12/214 (6)

[a] Log_{10} mosquito infective dose 50 per ml

[b] Number mosquitoes positive for dengue-2 viral antigen in midgut/number tested.

[c] Number mosquitoes positive for dengue-2 viral antigen in head tissues/number tested.

Table 32. Infection and transmission rates for Aedes albopictus (OAHU) orally infected with dengue-2 parent (PR-159) and attenuated (S-1) viruses.[a]

	Dengue-2 virus	
	Parent	Vaccine
No. mosquitoes exposed	22	29
No. infected (%)	22 (100)	16 (55)
No. disseminated[b]	22 (100)	8 (28)
No. transmitting (%)	15 (75)	9 (56)

[a] Dengue-2 viruses were grown in LLC-MK_2 cells at 31°C; post-feeding titer was 7.2 $\log_{10}MID_{50}$/ml for PR 159 and S-1 viruses.

[b] Dengue-2 viral antigen detected in mosquito head tissues.

Table 33. Plaquing of dengue-2 parent (PR-159) and attenuated (S-1) viruses at permissive and restrictive temperatures before and after oral passage in Aedes aegypti mosquitoes.

No.	Sample	PFU[a]/0.2 ml 35°C	38.5°C	39.3°C
1.	S-1 grown in C_6/36 cells at 27°C	5.1×10^5	4.4×10^3	<10
2.	S-1 from Ae. aegypti orally infected with #1	2.2×10^4	2.8×10^2	<10
3.	S-1 grown in LLC-MK_2 cells at 31°C	5.1×10^3	1.7×10^2	<10
4.	S-1 from Ae. aegypti orally infected with #3	4.9×10^3	3.0×10^1	<10
	S-1 control	3.4×10^5	2.3×10^3	<10
5.	Parent grown in C_6/36 cells at 27°C	4.5×10^6	1.3×10^5	8.4×10^3
6.	Parent from Ae. aegypti orally infected with #5	1.0×10^3	<10	<10
	Parent control	1.7×10^6	6.7×10^5	3.2×10^5

[a] Samples 1-6 all contained dengue-2 virus that resulted in heterogeneous plaque sizes ranging from 0.5 mm to 1.5 mm, except sample #5, which contained plaques that were 2.0 mm in size. None of the samples (1-6) contained the large plaques seen in the PR 159 control.

Table 34. Infection rates for Aedes aegypti and Aedes albopictus orally infected with graded doses of dengue-2 parent (PR-159) and vaccine (S-1) viruses.

Titer of feeding suspension $\log_{10}TCID_{50}$/ml	Parent (PR-159)		Vaccine (S-1)	
	Aedes aegypti	Aedes albopictus	Aedes aegypti	Aedes albopictus
8.2 - 7.2	3 (100)[a]	15 (100)	8 (100)	20 (5)
7.2 - 6.2	21 (95)	25 (100)	25 (28)	17 (65)
6.2 - 5.2	20 (65)	18 (95)	29 (7)	14 (57)
5.2 - 4.2	24 (25)	11 (91)	26 (4)	11 (18)
Total				
8.2 - 4.2	68 (66)	70 (97)	88 (20)	62 (65)

[a] Number tested (percent positive).

Table 35. Infection and dissemination rates of Aedes albopictus mosquitoes orally infected with graded doses of dengue-3 parent and candidate vaccine virus(CH53489, Clone 24/28)[a].

Blood Meal[b] Dilution	PARENT VIRUS Infected[c]	PARENT VIRUS Disseminated[d]	VACCINE VIRUS Infected	VACCINE VIRUS Disseminated
10^{0}	61/61 (100)	53/57 (93)	36/36 (100)	24/34 (71)
10^{-1}	52/53 (93)	46/49 (94)	48/50 (96)	45/50 (90)
10^{-2}	34/34 (100)	28/34 (82)	33/36 (92)	25/36 (69)
10^{-3}	42/53 (79)	33/51 (65)	32/40 (80)	23/38 (61)
Total	189/201 (94)	160/191 (84)	149/162 (92)	117/158 (74)

[a] Mosquitoes were maintained at 27°C during extrinsic incubation.

[b] Parent and vaccine blood meal titers 8.4 and 8.2 $\log_{10}$ $TCID_{50}$ per ml respectively.

[c] Number mosquitoes positive for dengue-3 viral antigen in abdominal tissues/number tested (%).

[d] Number mosquitoes positive for dengue-3 viral antigen in head tissues/number tested (%).

Table 36. Infection and dissemination rates of Aedes aegypti mosquitoes orally infected with graded doses of dengue-3 parent and candidate vaccine virus (CH53489, Clone 24/28)[a].

Blood Meal[b] Dilution	PARENT VIRUS Infected[c]	PARENT VIRUS Disseminated[d]	VACCINE VIRUS Infected	VACCINE VIRUS Disseminated
10^0	30/30 (100)	30/30 (100)	14/14 (100)	14/14 (100)
10^{-1}	27/27 (100)	27/27 (100)	27/27 (100)	27/27 (100)
10^{-2}	22/24 (92)	21/24 (88)	18/18 (100)	17/18 (94)
10^{-3}	11/18 (61)	10/18 (56)	12/21 (57)	10/21 (48)
Total	90/99 (91)	88/99 (89)	71/80 (89)	68/80 (85)

[a] Mosquitoes were maintained at 27°C during extrinsic incubation.

[b] Parent and vaccine blood meal titers 7.8 $\log_{10}$ $TCID_{50}$ per ml each.

[c] Number mosquitoes positive for dengue-3 viral antigen in abdominal tissues/number tested (%).

[d] Number mosquitoes positive for dengue-3 viral antigen in head tissues/number tested (%).

Table 37. Replication of dengue-3 parent and vaccine (CH53489, Clone 24/28) viruses in Aedes albopictus mosquitoes after oral infection and extrinsic incubation at 27°C.

		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	≥5.4	3.8	2.7	4.6	2.3	3.2	3.1	3.8	4.3	3.6
	2	≥5.8	Tr[b]	2.2	4.4	3.6	3.9	3.8	3.8	3.8	3.9
	3	≥5.7	4.3	4.1	4.1	3.6	3.9	3.1	4.2	3.9	4.1
	4	4.8	4.0	2.2	4.1	3.5	3.8	4.1	4.2	3.8	4.1
Mean		≥5.4	3.3	2.8	4.3	3.3	3.7	3.5	4.0	4.0	3.9
Vaccine	1	≥5.3	Tr	2.7	3.8	≥4.4	4.9	4.7	2.9	3.7	3.8
	2	≥5.6	4.3	3.8	3.7	5.2	4.2	4.6	3.2	3.9	2.8
	3	≥5.8	4.0	3.1	3.8	3.7	4.4	5.2	3.2	3.2	3.7
	4	≥4.8	4.1	3.1	0	3.8	4.6	4.6	3.1	3.7	3.7
Mean		≥5.4	3.4	3.2	3.8	≥4.3	4.5	4.8	3.1	3.6	3.5

[a] Blood meal titers: Parent virus 7.8 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.3 $\log_{10}$ $TCID_{50}$ per ml

[b] Tr = Trace

Table 38. Replication of dengue-3 parent and vaccine (CH53489, Clone 24/28) viruses in Aedes albopictus mosquitoes after oral infection and extrinsic incubation at 33°C.

		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	≥5.4	4.5	Tr[b]	4.3	≥5.7	≥5.3	4.5	≥5.1	≥5.3	5.1
	2	≥5.8	4.1	4.1	5.2	5.2	5.1	4.6	5.0	≥5.4	≥5.8
	3	≥5.7	Tr	5.2	5.2	≥5.8	≥5.8	5.3	5.1	≥5.7	≥5.2
	4	4.8	0	4.7	5.7	≥4.8	4.9	≥4.8	5.0	≥4.8	≥5.6
Mean		≥5.4	3.2	3.7	5.1	≥5.4	≥5.3	≥4.8	≥5.0	≥5.3	≥5.4
Vaccine	1	≥5.3	4.8	4.6	4.9	5.2	≥5.4	≥5.8	≥5.3	4.9	4.9
	2	≥5.6	4.0	4.7	5.3	5.3	≥5.8	5.2	≥5.7	≥5.6	5.1
	3	≥5.8	5.0	5.0	4.0	5.2	≥5.7	5.1	≥5.8	5.2	4.9
	4	≥4.8	4.5	≥4.6	≥4.7	4.3	≥4.8	≥5.6	≥4.8	≥5.6	4.9
Mean		≥5.4	4.6	≥4.7	≥4.7	5.0	≥5.4	≥5.4	≥5.4	≥5.3	≥5.0

[a] Blood meal titers: Parent virus 7.8 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.3 $\log_{10}$ $TCID_{50}$ per ml

[b] Tr = Trace

Table 39. Replication of dengue-3 parent and vaccine (CH53489, Clone 24/28) viruses in Aedes aegypti mosquitoes after oral infection and extrinsic incubation at 27°C.

		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	4.8	3.5	4.7	4.2	4.8	4.9	4.1	4.8	5.1	4.8
	2	5.0	3.5	4.0	3.8	4.8	4.8	4.7	4.6	4.8	5.2
	3	5.1	3.1	≥4.6	4.1	4.0	4.4	4.6	4.7	5.2	5.2
	4	4.8	0	4.6	≥5.3	4.6	4.8	5.0	5.0	4.6	4.3
Mean		4.9	3.4	≥4.5	≥4.4	4.6	4.7	4.6	4.8	4.9	4.9
Vaccine	1	4.6	3.6	3.2	3.8	4.5	4.1	4.2	4.3	3.9	4.6
	2	5.1	4.3	4.7	4.1	3.6	5.1	4.5	4.8	≥5.2	≥5.1
	3	5.3	3.5	4.2	3.2	4.6	4.3	≥4.4	4.6	≥4.8	≥5.4
	4	5.0	3.3	4.0	5.1	4.8	≥5.2	≥5.5	5.1	5.0	4.8
Mean		5.0	3.7	4.0	4.1	4.4	≥4.7	≥4.7	4.7	≥4.7	≥5.0

[a] Blood meal titers: Parent virus 6.8 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 6.8 $\log_{10}$ $TCID_{50}$ per ml

Table 40. Replication of dengue-3 parent and vaccine (CH53489, Clone 24/28) viruses in Aedes aegypti mosquitoes after oral infection and extrinsic incubation at 33°C.

		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	4.8	3.1	4.0	4.5	4.3	4.8	≥5.0	≥5.1	3.8	4.0
	2	5.0	4.1	4.0	5.1	4.6	5.1	4.8	4.8	3.5	3.6
	3	5.1	2.4	4.1	4.5	4.0	4.8	≥4.9	4.6	3.1	4.3
	4	4.8	3.8	≥5.3	4.6	4.5	5.0	4.8	4.1	4.1	3.1
Mean		4.9	3.4	≥4.2	4.7	4.4	4.9	≥4.9	≥4.7	3.6	3.8
Vaccine	1	4.6	3.8	4.8	5.1	4.6	4.6	4.6	3.8	3.8	4.3
	2	5.1	3.1	4.3	≥5.6	4.0	4.5	4.8	4.1	4.0	3.8
	3	5.3	3.2	5.0	≥5.5	4.1	4.3	4.6	4.0	4.6	4.3
	4	5.0	3.0	≥5.3	≥5.6	4.5	3.6	4.6	4.5	4.1	4.0
Mean		5.0	3.3	≥4.9	≥5.5	4.3	4.3	4.7	4.1	4.1	4.1

[a] Blood meal titers: Parent virus 6.8 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 6.8 $\log_{10}$ $TCID_{50}$ per ml

Table 41. Infection, dissemination, and transmission rates of Aedes albopictus mosquitoes engorging a blood meal of dengue-3 parent or candidate vaccine (CH53489, Clone 24/28) virus[a] and extrinsically incubated for 14 or 21 days at 27°C or 33°.

		PARENT		VACCINE	
		14 Days	21 Days	14 Days	21 Days
27°C	Infection	14/14 (100)	36/38 (95)[b]	11/11 (100)	58/59 (98)
	Dissemination[c]	7/11 (64)	35/38 (92)	7/8 (88)	45/57 (79)
	Transmission	ND[d]	0/20 (0)	1/20 (5)	2/20 (10)
33°C	Infection	15/16 (94)	15/15 (100)	18/18 (100)	6/6 (100)
	Dissemination	4/15 (27)	14/15 (93)	9/17 (53)	6/6 (100)
	Transmission	ND	0/20 (0)	ND	0/20 (0)

[a] Blood meal titers: Parent virus 7.8 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.3 $\log_{10}$ $TCID_{50}$ per ml

[b] Number of mosquitoes positive for viral antigen/Number tested (%)

[c] Dissemination rates may be low due to a masking effect by the immersion oil.

[d] ND = Not Done

Table 42. Infection, dissemination, and transmission rates of Aedes aegypti mosquitoes engorging a blood meal of dengue-3 parent or candidate vaccine (CH53489, Clone 24/28) virus[a] and extrinsically incubated for 14 or 21 days at 27°C or 33°C.

		PARENT		VACCINE	
		14 Days	21 Days	14 Days	21 Days
27°C	Infection	ND	42/43 (98)[b]	ND	24/25 (96)
	Dissemination	ND	24/26 (92)[c]	ND	12/12 (100)
	Transmission	ND	0/18 (0)	ND	2/20 (10)
33°C	Infection	ND	32/34 (94)	ND	ND[d]
	Dissemination	ND	18/21 (86)	ND	ND
	Transmission	ND	2/20 (10)	ND	0/11 (0)

[a] Blood meal titers: Parent virus 6.8 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 6.8 $\log_{10}$ $TCID_{50}$ per ml

[b] Number of mosquitoes positive for viral antigen/Number tested (%)

[c] Dissemination rates of mosquitoes exposed to immersion oil were excluded due to a possible masking effect.

[d] ND = Not Done

Table 43. Plaquing of dengue-3 attenuated virus (CH53489, Clone 24/28) at permissive and restrictive temperatures after 3 passages in Aedes albopictus or aegypti mosquitoes.

	PFU/ml			
Sample	31°C	35°C	38.5°C	39.3°C
Clone 24/28 passaged in Aedes albopictus				
Pool 1[a]	1.5×10^5(S)[b]	2.9×10^4(S)	$<5 \times 10^1$	<5
Pool 2	5.5×10^4(S)	3.1×10^4(S)	$<5 \times 10^1$	<5
Clone 24/28 passaged in Aedes aegypti				
Pool 1	2.5×10^5(S)	5.0×10^4(S)	$<5 \times 10^1$	<5
Pool 2	7.5×10^4(S)	5.5×10^4(S)	$<5 \times 10^1$	<5
Clone 24/28 Control	1.8×10^5(S)	5.5×10^4(S)	5.8×10^3(S)	1.7×10^2(S)

[a] Five mosquitoes were triturated in 2 ml of diluent, aliquoted, and stored at -70°C until analysis.

[b] Samples contained virus of small plaque morphology of < 1.0 mm.

Table 44. Infection rates of Aedes albopictus mosquitoes orally infected with graded doses of dengue-4 parent (H-241) and vaccine (H-214, Lot 1) viruses.[a]

Titer of Blood Meal[b]	Parent Virus		Vaccine Virus	
	Infected (%)	Disseminated (%)	Infected (%)	Disseminated (%)
7.0[b]	19/26[c] (73)	16/26[d] (62)	7/29 (24)	6/29 (21)
6.0	51/71 (72)	48/71 (68)	9/76 (12)	6/76 (0)
5.0	17/48 (35)	11/48 (23)	0/36 (0)	0/36 (0)
4.0	6/41 (15)	4/41 (10)	0/13 (0)	0/13 (0)

[a] Mosquitoes were incubated at 27°C after engorgement.

[b] $Log_{10}TCID_{50}$ per ml.

[c] Number of mosquitoes with detectable dengue-4 antigen in the midgut/number examined.

[d] Number of mosquitoes with detectable dengue-4 antigen in head tissues/number examined.

Table 45. Replication of dengue-4 parent (H-241) and vaccine (H-241, Lot 1) viruses in Aedes albopictus mosquitoes after oral infection[a].

		Days post infection									
Virus[a]	Mosquito	0	3	5	7	9	11	14	16	19	21
Parent	1	NT[b]	TR[c]	0	3.0	3.3	4.8	4.0	5.0	5.0	0
	2	NT	0	4.3	TR	0	4.3	3.8	4.8	5.3	3.3
	3	NT	0	3.0	2.8	2.5	3.0	3.8	4.8	5.3	3.3
	4	NT	0	5.3	0	3.8	3.3	4.3	4.5	3.3	3.3
Mean Titer		NT	0.3	3.1	1.7	2.4	3.8	4.1	4.7	3.4	2.6
Vaccine	1	NT	0	0	0	0	0	0	2.3	0	TR
	2	NT	0	0	TR	0	0	0	2.0	0	3.3
	3	NT	0	0	0	0	0	0	3.3	0	0
	4	NT	0	0	0	0	3.5	0	TR	0	0
Mean Titer		NT	0	0	0.3	0	0.9	0	2.1	0	1.1

[a] Dengue-4 parent virus bloodmeal titered 9.5 $\log_{10}TCID_{50}$ per ml. Dengue-4 vaccine virus bloodmeal titered 7.0 $\log_{10}TCID_{50}$ per ml.

[b] NT = not tested.

[c] TR = trace.

Table 46. Replication of dengue-4 parent (H-241) and vaccine (H-214, Lot 1) viruses in Aedes aegypti mosquitoes after oral infection and extrinsic incubation at 27°C.

		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	4.0	0	0	4.3	≥4.5	4.5	≥5.5	ND[c]	≥4.5	0
	2	3.3	0	3.8	3.8	4.8	4.2	5.5	ND	4.5	0
	3	4.3	Tr[b]	2.0	3.5	0	≥5.0	≥5.3	ND	0	≥5.5
	4	3.8	0	3.0	≥4.0	3.5	4.5	≥4.0	ND	≥5.0	≥5.5
Mean		3.9	0.3	2.2	≥3.9	≥3.2	≥4.6	≥5.1	--	≥3.5	≥4.0
Vaccine	1	3.3	0	Tr	0	0	0	0	0	2.8	2.8
	2	3.8	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0
	4	2.8	0	Tr	0	≥5.5	3.0	0	0	0	0
Mean		2.5	0	0.5	0	≥1.4	0.8	0	0	0.2	0.2

[a] Blood meal titers: Parent virus 7.0 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 7.0 $\log_{10}$ $TCID_{50}$ per ml

[b] Tr = Trace

[c] ND = Not Done

Table 47. Replication of dengue-4 parent (H-241) and vaccine (H-241, Lot 1) viruses in Aedes albopictus mosquitoes after oral infection[a] and extrinsic incubation at 30°C.

		Titer of virus ($TCID_{50}$)									
		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	4.0	3.8	3.3	≥3.0	≥4.3	4.5	≥5.5	ND[b]	2.3	≥4.5
	2	3.3	2.5	4.0	≥5.0	3.8	0	4.0	ND	≥5.5	0
	3	4.3	3.5	4.8	3.3	0	4.3	5.0	ND	≥5.5	≥5.0
	4	3.8	4.5	3.5	0	≥5.5	3.3	0	ND	≥5.5	0
Mean Titer		3.9	3.6	3.9	2.8	3.4	3.0	3.6	--	≥4.7	≥2.4
Vaccine	1	3.3	0	ND	0	0	3.8[c]	0	0	0	0
	2	3.8	0	ND	0	2.5	3.8	0	0	TR[d]	0
	3	0	0	ND	0	TR	2.0	0	0	0	0
	4	2.8	0	ND	0	0	4.0	0	0	0	0
Mean Titer		2.5	0	ND	0	0.9	3.4	0	0	0.3	0

[a] Dengue-4 parent and vaccine virus bloodmeals titered 7.0 $\log_{10}TCID_{50}$ per ml.

[b] ND = Mosquitoes are currently being processed.

[c] Probable sampling error, mosquitoes to be tested to determine if infected with parent or vaccine virus.

[d] TR = trace.

Table 4. Comparison of geometric mean titers of Aedes albopictus mosquitoes [illegible] infected with dengue-4 viruses.

Virus type				Days post infection							
				5	7	9	11	14	17	19	21
Parent	[illegible]	[illegible]	[illegible]	[illegible]	1.9	3.2	4.6	5.1	ND[a]	3.5	4.0
	30°C	[illegible]	[illegible]	1.9	2.8	3.4	3.0	3.6	ND	4.7	2.4
Vaccine	[illegible]	[illegible]	0	0.3	0	1.4	0.8	0	0	0.7	0.7
	30°C	2.4	0	ND	0.9	3.4[b]	0	0	0.3	0	2.6

[a] ND = not done.

[b] Probably sampling error, mosquitoes to be tested to determine if infected with parent or vaccine virus.

Table 49 Replication of dengue-4 parent and vaccine (H-241, Lot 1) viruses in Aedes albopictus mosquitoes after oral infection and extrinsic incubation at 27°C.

		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	≥5.5	3.5	≥5.5	4.5	4.5	4.3	5.0	3.8	≥5.3	4.8
	2	≥5.5	2.0	4.8	4.8	4.5	4.5	≥5.3	4.5	4.8	5.0
	3	5.0	3.5	≥5.5	≥5.5	4.5	4.5	3.5	3.0	3.8	≥5.5
	4	5.0	0	3.3	5.0	≥5.3	2.0	3.5	3.5	3.3	4.0
Mean		≥5.3	2.3	≥4.8	≥5.0	≥4.7	3.8	≥4.3	3.7	≥4.3	≥4.8
Vaccine	1	5.0	0	0	Tr[b]	0	0	3.3	2.8	0	0
	2	4.3	0	0	0	3.0	0	2.8	0	0	Tr
	3	5.0	0	0	2.8	2.3	0	0	≥5.3	0	0
	4	4.5	0	0	2.8	3.3	2.3	0	0	3.3	0
Mean		4.7	0	0	1.7	2.2	(2.3) 0.6	1.5	≥2.0	(3.3) 0.8	(1.0) 0.3

[a] Blood meal titers: Parent virus 9.0 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.5 $\log_{10}$ $TCID_{50}$ per ml

[b] Tr = Trace

Table 50. Replication of dengue-4 parent and vaccine (H-241, Lot 1) viruses in Aedes albopictus mosquitoes after oral infection and extrinsic incubation at 33°C.

		Days Post-Feeding									
Virus[a]	Mosquito	0	3	5	7	9	11	14	17	19	21
Parent	1	≥5.5	3.3	4.5	4.5	4.8	4.5	4.8	4.0	≥5.3	≥5.3
	2	≥5.5	0	ctm[b]	4.0	3.8	ctm	3.8	≥5.5	0	≥5.5
	3	5.0	2.5	ctm	5.0	4.8	4.5	4.8	≥5.5	≥5.5	≥5.5
	4	5.0	2.8	3.8	5.0	3.5	2.5	4.5	≥4.5	≥5.5	5.5
Mean		≥5.3	2.2	4.2	4.6	4.2	3.8	4.5	≥4.9	≥5.4	≥5.5
Vaccine	1	5.0	ND[c]	ND	ND	0	0	0	0	1.8	0
	2	4.3	ND	ND	ND	0	4.0	≥5.0	0	0	0
	3	5.0	ND	ND	ND	0	2.3	0	5.0	0	4.5
	4	4.5	ND	ND	ND	3.8	0	0	0	3.5	0
Mean		4.7	-	-	-	1.0	1.6	≥1.3	≥1.3	1.3	1.1

[a] Blood meal titers: Parent virus 9.0 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.5 $\log_{10}$ $TCID_{50}$ per ml

[b] ctm = contaminated

[c] ND = not done

Table 51. Infection rates of Aedes albopictus mosquitoes orally infected with dengue-4 parent (H-241) and vaccine (H-241, Lot 1) viruses after extrinsic incubation periods of 14 or 21 days at 27°C or 30°C.

Length of Extrinsic Incubation	Parent Virus		Vaccine Virus	
	27°C	30°C	27°C	30°C
14 days	8/9 (89%)	7/9 (78%)	0/9 (0%)	1/9 (11%)
21 days	22/30 (73%)	14/18 (78%)	8/33 (24%)	0/17 (0%)
Total	30/39 (77%)	21/27 (78%)	8/42 (19%)	1/26 (4%)

Table 52. Pathogenesis of dengue-4 viruses in Aedes albopictus mosquitoes after oral infection and incubation for 21 days at 27°C and 30°C.

			TISSUES					
Virus	Temp	Mosquito	Head	Ganglion	Salivary Glands	Ovarial Sheath	Gut Mid	Tissues Hind
PARENT	27°C	1	-	-	-	-	-	-
		7	+	-	-	-	4+	-
		11	2+	-	-	-	3+	-
		12	3+	2+	4+	2+	4+	-
		13	-	-	-	-	-	-
	30°C	1	4+	3+	4+	3+	4+	+
		3	4+	3+	3+	4+	M[a]	M
		4	-	-	-	-	-	-
		7	4+	4+	2+	4+	-	-
VACCINE	27°C	1	-	-	-	-	-	-
		3	-	-	-	-	-	-
		4	-	-	-	-	-	-
		11	-	-	-	-	-	-
		13	-	-	-	-	-	-
	30°C	3	-	-	-	-	-	-
		4	-	-	-	-	-	-
		5	-	-	-	-	-	-
		9	-	-	-	-	-	-
		11	-	-	-	-	-	-

[a] = Missing.

Table 53. Infection, dissemination, and transmission rates of Aedes albopictus mosquitoes engorging a blood meal of dengue-4 parent (H-241) or vaccine (H-241, Lot 1) virus[a] and extrinsically incubated at 27°C or 33°C.

		Parent		Vaccine	
		7 days[b]	14 days	7 days	14 days
27°C	Infection	0/10 (0)[c]	7/10 (70)	0/10 (0)	0/10 (0)
	Dissemination	0/10 (0)	7/10 (70)	0/10 (0)	0/10 (0)
	Transmission[d]	-	ND[4]	-	-
33°C	Infection	3/10 (30)	10/10 (100)	0/10 (0)	0/7 (0)
	Dissemination	1/10 (10)	10/10 (100)	0/10 (0)	0/7 (0)
	Transmission	ND	ND	-	-

[a] Blood meal titers: Parent virus 7.50 $\log_{10}TCID_{50}$/ml
Vaccine virus 7.25 $\log_{10}TCID_{50}$/ml

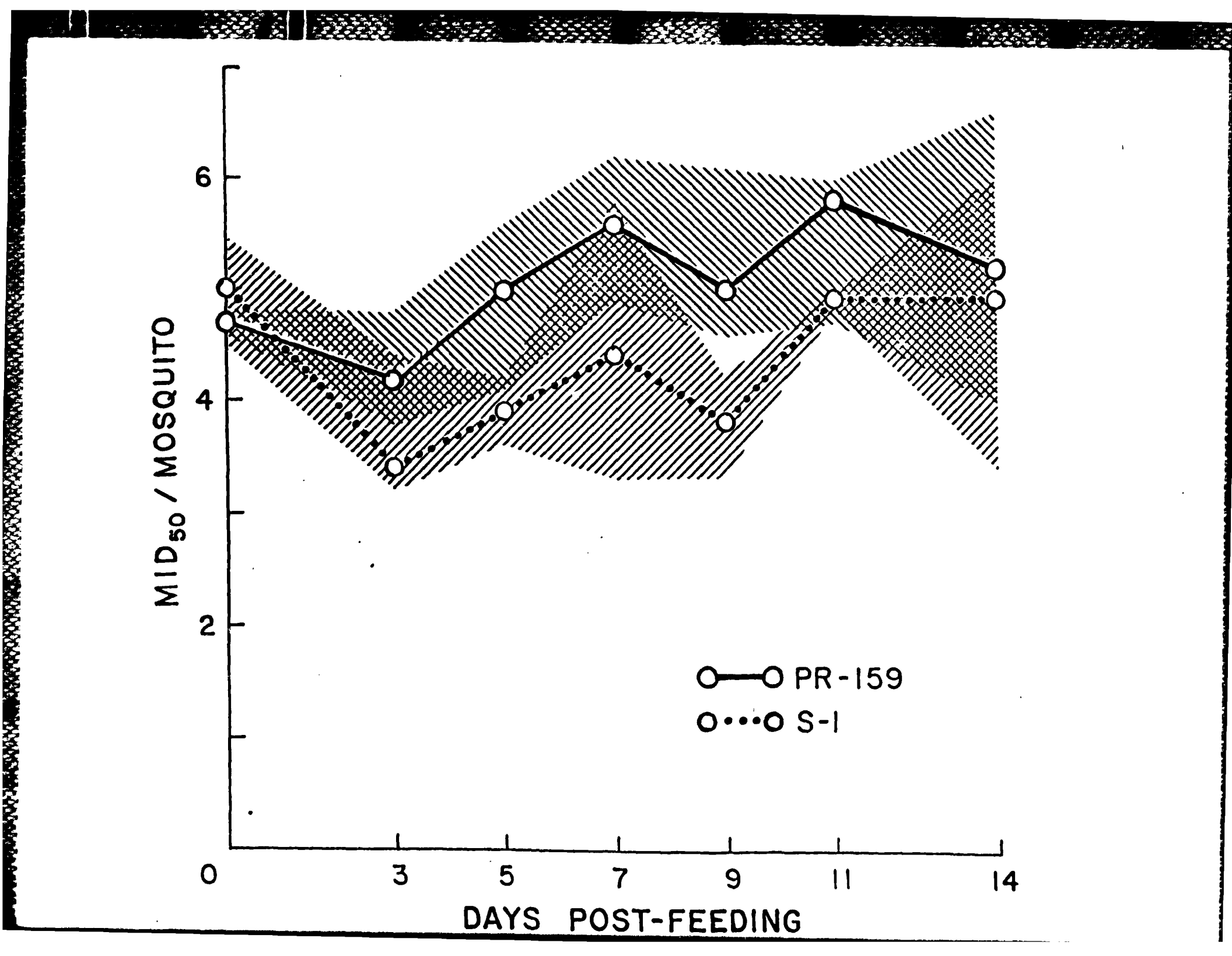
[b] Days extrinsic incubation post-infection.

[c] Number positive/total (%)

[d] ND = Not Done

Figure 1

Replication of dengue-2 parent (PR-159) and vaccine (S-1) viruses in Aedes aegypti mosquitoes.[a,b] [a]Crosshatched area indicates range of titers. [b]Dengue-2 viruses were grown in LLC-MK_2 cells at 31°C; post-feeding titer of the bloodmeals were 7.2 MID_{50} per ml for both the parent and vaccine viruses.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MID50 / MOSQUITO
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DAYS POST-FEEDING

AD-A167 990 PATHOGENESIS OF DENGUE VACCINE VIRUSES IN MOSQUITOES (U) COLORADO STATE UNIV FORT COLLINS COLL OF VETERINARY MEDICINE AND BIOMEDICAL SCIENCES B J BEATY 01 JUL 85 2/2

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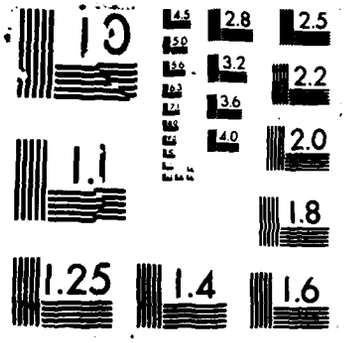
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